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Studies on the Effect of Vitamins in the Animals under the Cold

Part I. Effect of Cold Stress in Growth and the Vitamin A Content of the Kidney of Rats

By Tatsuo KOYANAGI and Satoshi ODAGIRI

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Received January 25, 1957

Some evidences have indicated that the cold environmental temperature has a depressing effect on growth and the vitamin A contained in the kidney of rats. The supplement of the vitamin A revealed to counteract the depressing effect on growth.

During a long term-investigation of the plasma vitamin A level of cows, the authors found a decrease occuring at the beginning of winter. So they presumed that an increase in the destruction of vitamin A in cows may occur, when they were subjected to a low temperature. As for the relation between vitamin A and resistance to cold, Grab et al.[1] and Ershoff[2], reported that there was an impaired resistance of the rat to cold stress due to the deficiency of vitamin A.

In the present communication, data on the effects of cold on growth and the vitamin A content of the kidney of rats are presented.

EXPERIMENTAL

Twelve rats of the wister strain weighing about 90 g, were divided into 3 groups-A, B and C each consisting of 4 animals fed with 10 g of a basal ration which had the following composition: rice powder 62%, skim-milk powder 30%, soybean oil 3%, beer yeast 3%, table salt 1% and calcium carbonate 1%. Rats in groups A and B were in addition, administrated oral supplements of vitamin A concentrate in amounts furnishing a daily intake of 1 γ during the two preceding weeks. Thereafter, they were not supplemented. The litters in group C received 2 γ of vitamin A per body weight in the two preceding weeks, and 1 γ in the next two weeks. Since the beginning of the experiment the cages of groups C and B were placed in the cold room (0–5°C). The cages of group A were held at a temperature range of 20–23°C.

At the end of four weeks, all the animals were sacrificed for decapitation and the vitamin A of the kidney, ascorbic acid and succinic oxidase of the liver were determined. The authors selected the kidney as the material of analysis because Johnson and Baumann[3] reported that when rats were kept on a diet deficient in vitamin A, more vitamin A was accumulated in the kidney than in the liver.

Vitamin A was extracted from the tissue after digestion with alkali, and measured by the glycerol dichlorohydrin method[4,5], with the use of a photoelectric absorptiometer. The reduced form of ascorbic acid of the liver was determined according to the indophenol-method. Succinic oxidase activity was determined by the manometric method using the homogenate of the livers prepared by the method of Potter and Elvehjem[6].

RESULTS AND DISCUSSION

The growth of animals placed in the cold room was significantly retarded and when vitamin A was supplemented the growth rate somewhat recovered, whereas, the animals in the warm room continued to grow most favorably among the three groups.

---

[1] W. Grab und K. Lang, *Klin. Wchnschr.*, 21/26, 230 (1944).

[2] B.H. Ershoff, *Proc. Soc. Exp. Biol. Med.*, **74**, 586 (1950).

[3] R.M. Johnson and C.A. Baumann, *Arch. Biochem.*, **14**, 361 (1947); *Fed. Proc.*, **6**, 265 (1947).

[4] A.E. Sobel and H. Werbin, *Anal. Chem.*, **19**, 107 (1947).

[5] A. Fujita and M. Aoyama, *Vitamins*, **4**, 127 (1951); **6**, 230 (1953).

[6] V. D. Potter and C.A. Elvehjem, *J. Biol. Chem.*, **114**, 495 (1936).

TABLE I

GROWTH RATE, VITAMIN A CONTENT OF THE KIDNEYS, ASCORBIC ACID CONTENT AND SUCCINIC OXIDASE ACTIVITY OF THE LIVER OF RATS PLACED IN THE WARM OR COLD ROOMS

Group and temp. of rooms °C	Vit. A per day per body γ	No. of rats	Initial body wt. g	Gain in body wt. 4 weeks g	Vit. A in kidney γ%	Ascorbic acid in liver mg%	Succinic oxidase activity*** O_2 μl
A 20–23	1*	2 ♀	95	52	569	9.8	54.6
		3 ♂	98	65	625	—	—
		8 ♀	83	31	875	11.5	66.0
		9 ♀	87	37	665	10.7	63.6
		mean	90.7	46.3	684	10.7	61.4
B 0–5	1*	1 ♀	86	29	351	11.7	—
		7 ♂	97	27	221	18.0	58.7
		13 ♂	91	20	252	17.3	56.1
		14 ♂	100	33	157	16.6	46.0
		mean	93.5	27.2	245	15.9	53.6
C 0–5	2**	4 ♀	100	30	301	10.4	49.3
		6 ♂	91	44	299	14.2	46.4
		11 ♂	84	56	157	11.1	49.9
		12 ♂	85	26	315	15.0	50.5
		mean	90.0	39.0	268	12.6	49.3

* Supplemented only during the initial 2 weeks.

** 2γ for the initial 2 weeks, thereafter, 1γ.

*** Amount of O_2 consumed during 60 min. with 40 mg of liver.

Data of analysis of vitamin A in Table I indicate that the levels of the vitamin contained in the kidney of rats kept at a low temperature are significantly depressed. The animals in the warm room had 684γ of vitamin A whereas those in the cold room had only 245γ per 100 g of kidney. Even the vitamin A supplemented animals (group C) had only 268γ of vitamin A. It therefore seemed that vitamin A in the body is consumed by cold. Namely, group C was lower in the level of vitamin A than the group A whereas, the former was supplemented twice of the amount of vitamin A. Rush7) reported that the hepatic storage of vitamin A of the female rats exceed those of the male rats. The same tendency was found in this experiment, but the difference in groups was found to be more larger than sex.

At the end of the experimental period, no external symptoms of vitamin A deficiency appeared, except that the cold room groups were considerably slow in motion.

Autopsy revealed that all the animals of groups of the cold series were found to have congested lungs, whereas those of the warm room group were found to be normal.

The ascorbic acid content of liver, in contrast with the vitamin A content, was elevated in the animals in the cold room.

Succinic oxidase activity was higher in the animals of the warm room than the two other groups. This was in accordance with the better growth rate of the warm room group. There was no difference in the activity between the two groups kept in the cold temperature.

Results of the present experiment indicate that vitamin A which was sufficient to promote growth but suboptimal growth, in the warm place is inadequate for optimal resistance to stress caused by prolonged exposure to a low environmental temperature.

The nature of this apparent effect of low temperature on vitamin A of the kidney, observed in this experiment is not known. The utilization of the vitamin may have increased, or the absorption of vitamin A from the gastrointestinal tract may have decreased, or

---

7) B. Rush and B.L. Truscott, *Endocrinology*, **49**, 461 (1951).

both may have taken place. Increase of utilization of the vitamin can be considered because the vitamin has a close relation to pituitary and adrenals, where hormones concerning the regulation of body temperature and growth are produced.

As for ascorbic acid the problem is complicated, because the rat can produce this vitamin inside its own body. Squibb[8] reported that when rats are subjected to a high temperature for 72 hrs. the levels of vitamin A and ascorbic acid in their blood serum were significantly depressed.

The results of these experiments suggest the need of vitamin A and ascorbic acid for growth and metabolism in animals.

Acknowledgement: The authors are indebted to the *Nihon Eisai Co.* for the generous gift of the vitamin A concentrate. This work was aided by a grant from the Scientific Research Fund, Ministry of Education.

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[8] R.I. Squibb, M.A. Guzman and N.S. Scrimshaw, *J. Nutrition*, **52**, 155 (1954).

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Microbiological Studies of *Coli-aerogenes* Bacteria

Part I. Conversion of the Lactic Acid Fermentation to α-Ketoglutaric Acid Fermentation

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Received January 25, 1957

During investigations on the metabolisms of glucose by *coli-aerogenes* bacteria, it was found that the bacteria accumulated a large amount of α-ketoglutaric acid under aerobic conditions such as shaking culture, while lactic acid was ascertained to be produced anaerobically by the bacteria as was already known.

INTRODUCTION

It is a well-known fact that the bacteria of the genus *Escherichia* and genus *Aerobacter* produce lactic, acetic, formic and succinic acids, ethyl alcohol, acetylmethylcarbinol, 2:3 butyleneglycol, CO_2 and H_2 by the anaerobic fermentation of glucose.

The occurrence of lactic dehydrogenase is observed in general with microorganisms of *coli-aerogenes* types, so that lactic acid is produced by the reduction of pyruvic acid in the presence of the hydrogen donator (triose phosphate) as their main fermentation product[1]:

$$\mathrm{CH_3COCOOH + 2\,H \rightarrow CH_3CHOHCOOH}$$

The dismutation of pyruvic acid anaerobically takes place by the action of *E. coli*, according to the following equation[2,3]:

$$\mathrm{2\,CH_3COCOOH \rightarrow CH_3CHOHCOOH + CH_3COOH + CO_2}$$

Further, such a type of dismutation of pyruvic acid as an intramolecular dismutation, that occurs with *E. coli*[2,4], may be explained in the following way:

$$\mathrm{CH_3COCOOH + H_2O \rightarrow CH_3COOH + HCOOH}$$

Formic acid is further decomposed to H_2 and CO_2 by formic hydrogenlyase[5].

$$\mathrm{HCOOH \rightarrow H_2 + CO_2}$$

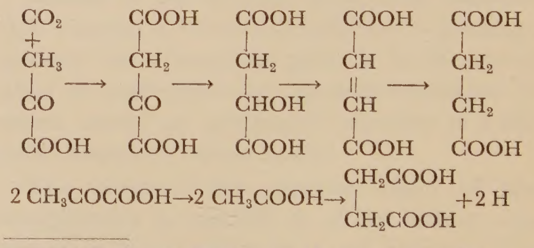
Moreover, acetylmethylcarbinol is formed from pyruvate by decarboxylation and condensation, with *Aerobacter aerogenes* as it will be seen in the following equation[6]:

$$\mathrm{2\,CH_3COCOOH \rightarrow CH_3COCHOHCH_3 + 2\,CO_2}$$

With some strains of *Aerobacter*, a reduction of acetylmethylcarbinol to 2 : 3 butyleneglycol[7] is observed:

$$\mathrm{CH_3COCHOHCH_3 + 2\,H \rightleftarrows CH_3CHOH \cdot CHOHCH_3}$$

Succinic acid would be formed in various stages of fermentation of pyruvate by the bacteria of *coli-aerogenes* types, and succinate is assumed to be produced at least by the two reactions illustrated as follows[8,9]:



$$\begin{array}{l}\mathrm{CO_2}\\+\\\mathrm{CH_3}\\|\\\mathrm{CO}\\|\\\mathrm{COOH}\end{array}\longrightarrow\begin{array}{l}\mathrm{COOH}\\|\\\mathrm{CH_2}\\|\\\mathrm{CO}\\|\\\mathrm{COOH}\end{array}\longrightarrow\begin{array}{l}\mathrm{COOH}\\|\\\mathrm{CH_2}\\|\\\mathrm{CHOH}\\|\\\mathrm{COOH}\end{array}\longrightarrow\begin{array}{l}\mathrm{COOH}\\|\\\mathrm{CH}\\\|\\\mathrm{CH}\\|\\\mathrm{COOH}\end{array}\longrightarrow\begin{array}{l}\mathrm{COOH}\\|\\\mathrm{CH_2}\\|\\\mathrm{CH_2}\\|\\\mathrm{COOH}\end{array}$$

$$\mathrm{2\,CH_3COCOOH \rightarrow 2\,CH_3COOH \rightarrow}\begin{array}{l}\mathrm{CH_2COOH}\\|\\\mathrm{CH_2COOH}\end{array}\mathrm{+2\,H}$$

---

[1] M. Stephenson, *Biochem. J.*, **22**, 605 (1928).

[2] H.A. Krebs, *Biochem. J.*, **31**, 2095 (1937).

[3] J. Tikka, *Biochem. Z.*, **279**, 264 (1935).

[4] C. Kalnitsky and C.H. Werkman, *Arch. Biochem.*, **2**, 113 (1943).

[5] M. Stephenson, *Ergeb. Enzym. forsch.*, **6**, 139 (1937).

[6] M. Silverman and C.H. Werkman, *J. Biol. Chem.*, **138**, 35 (1941).

[7] R.Y. Stanier and G.A. Adams, *Biochem. J.*, **38**, 168 (1944).

[8] S.R. Elsden, *Biochem. J.*, **32**, 187 (1938).

[9] T. Thunberg, *Skand. Arch. Physiol.*, 40, 1 (1920).

It is also ascertained that *E. coli* oxidizes pyruvic acid to acetic acid, under aerobic conditions[10,11]:

$$CH_3COCOOH + {}^1/_2O_2 \rightarrow CH_3COOH + CO_2$$

From the investigations mentioned above, it is suggested that the products of the carbohydrate breakdown revealed by *coli-aerogenes* bacteria vary with the type of their pyruvic acid metabolism.

In the course of investigating pyruvate metabolisms, the authors found an interesting phenomenon in which the microorganisms of *coli-aerogenes* types produced a large amount of α-ketoglutaric acid from glucose in growing culture under aerobic conditions.

In the present paper, lactic acid fermentation by the bacteria of *coli-aerogenes* types is observed to be converted into α-ketoglutaric acid fermentation under aerobic conditions.

EXPERIMENTAL AND RESULTS

Microorganisms Various strains of the bacteria of *coli-aerogenes* types, were used throughout the experiments.

Analytical Methods Lactic acid was determined by the method of Friedemann et al.[12], and ethanol by Kolthoff's method[13]. Acetic acid and acetylmethyl carbinol (acetoin) were estimated by the methods of Friedemann[14] and Van Niel, respectively[15]. Both pyruvic and α-ketoglutaric acids were determined by a modified method of Friedemann and Haugen[16]. Sugar determination was carried out in the usual manner by the Bertrand's method.

Statical Fermentation of Glucose For statical fermentation of glucose, one of the species of *E. coli*, strain No. G–2, was employed. The bacterium was grown in a medium containing 2.0% glucose, 0.1% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.5% NaCl and 0.05% $MgSO_4 \cdot 7H_2O$ in distilled water to which sterilized $CaCO_3$ in an amount of 75% of glucose used, was added at the time of inoculation. Eighty ml of the medium was dispensed in a 100-ml Erlenmeyer flask and it was sterilized. After inoculation, the medium was incubated at 28°C for 6 days under static conditions. The inoculum was prepared by suspending a loopfull of stock culture in 5 ml of bouillon and incubated at 37°C for 20 hours under statical conditions. About 0.4 ml of this bouillon culture was used as the inoculum. At the end of fermentation, the cultures were analysed; The results are shown in Table I.

From Table I, it is pointed out that lactic acid was the main product of static fermentation of glucose, and also, the formation of acetic acid, ethyl alcohol and pyruvic acid was detected from the analytical data.

Experiments of Surface Culture

In order to investigate whether there was a relationship of lactic acid to pyruvic acid, experiments of surface culture were carried out. The microorganisms of *coli-aerogenes* types which produce lactic acid as their main fermentation product are known to produce lactic dehydrogenase, so that it would be reasonable to assume that their formation of lactic acid is due to the reduction of pyruvic acid. Accordingly, it is expected that the production of lactic acid is decreased, and the formation of pyruvic acid as well as acetic acid is accelerated under aerobic conditions such as surface culture. The composition of the cultural medium used in this experiment was similar to the static cultures mentioned above, except the amount of $(NH_4)_2HPO_4$, which was a half (0.05%) of those used in the previous experiments.

Fernbach's flasks (1 l) containing 100 ml of the medium, and two species of *E. coli*, G–2 and S–1 were used, inoculated, and then incubated for 37°C for 4–8 days under static conditions. The results are

TABLE I

FERMENTATION PRODUCTS OF *E. COLI* UNDER STATIC CONDITIONS

	Glucose consumed	Lactic acid formed	Acetic acid formed	Pyruvic acid formed	Ethanol formed
mg/dl	2000.0	900.0	205.7	20.0	128.0
% on consumed glucose		45.0	10.3	1.0	6.4

---

[10] J.L. Still, *Biochem. J.*, **35**, 380 (1941).

[11] S. Korkes et al., *J. Biol. Chem.*, **193**, 721 (1951).

[12] T.E. Friedemann et al., *J. Biol. Chem.*, **73**, 335 (1927).

[13] L.M. Kolthoff, Die Massanalyse (Zweiter Teil), 329 (1928).

[14] T.E. Friedemann, *J. Biol. Chem.*, **123**, 161 (1938).

[15] Van Niel, *Biochem. Z.*, **187**, 472 (1927).

[16] T. E. Friedemann and G. E. Haugen, *J. Biol. Chem.*, **147**, 415 (1943).

TABLE II
FERMENTATION PRODUCTS BY A SURFACE CULTURE

Strains of *E. coli*	G–2 (4 days' incubation)			G–2 (8 days' incubation)	
Addition of riboflavin (mg/dl)	0	2.0	5.0	0	5.0
Glucose consumed (%)	72.2	77.2	84.9	86.8	99.6
Pyruvic acid produced (mg/dl)	49.8	43.8	58.2	40.2	34.5
Acetic acid produced (mg/dl)	168.0	168.0	264.0	204.0	240.0
α-Ketoglutaric acid produced (mg/dl)	40.2	38.9	53.9	38.9	38.9

shown in Table II in which pyruvate formation was found to be accelerated to a certain degree.

Besides pyruvic acid, a 2, 4-dinitrophenylhydrazone of some unknown keto acid, which was found to be difficultly soluble in toluol or xylol, was detected.

Paper Chromatography of Keto Acids of Surface Culture. At the end of the fermentation, culture liquor was filtered. The filtrate was mixed with a 2 N–HCl solution of 0.4% 2,4-dinitrophenylhydrazine. The mixture was kept at room temperature for 30 min. and then extracted with ethylacetate by bubbling a stream of air. The hydrazones were reextracted from ethylacetate into a small amount of 10% Na_2CO_3 and then spotted on sheets of filter paper (Toyo paper, No. 2). For the developing solvent, a mixture of 5 volumes of *n*-butanol, 1 volume of ethanol, and 4 volumes of water was applied[17], and they were developed by the ascending method.

It was found that the fermentation liquor of the surface culture gave two spots, corresponding to 2,4-dinitrophenylhydrazone of pyruvic acid, R_F 0.36, and the hydrazone of α-ketoglutaric acid, R_F 0.26.

Spectrophotometric Analysis. Moreover, the spectrophotometric analysis of keto acids was carried out. Keto acids were chromatographed on paper as described above. The bands on the paper corresponding to the hydrazones of pyruvate and α-ketoglutarate were independently cut out and the pieces of paper thus obtained, were eluted with a small amount of 10% Na_2CO_3. An equal volume of 1.5 N NaOH was added to the Na_2CO_3 solution, and the reddish color thus developed, was immediately measured spectrophotometrically on the wavelength between 350 and 600 mμ.

As it is shown in Figures 1 and 2, the results thus obtained are ascertained to agree quite well with the synthetic (authentic) acid.

From the results mentioned above, it was found that *E. coli* produced a small amount of α-ketoglutaric acid by surface culture. In the next place, the effect of riboflavin on the formation of α-ketoglutaric acid was tested by surface culture.

As it is shown in Table II, there was not any noticeable effect of riboflavin to be observed.

Experiments of Shaking Culture

As it was already shown in the previous experiment, *E. coli* produced a small amount of α-ketoglutaric acid besides pyruvic acid by the surface culture. Lockwood et al.,[18] found that *Pseudomonas fluorescens* produced

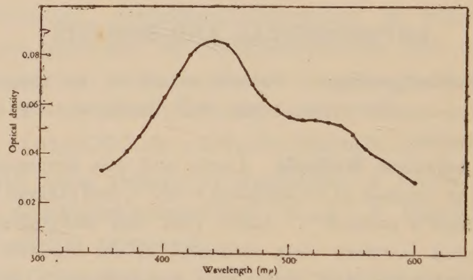


FIG. 1 Absorption Spectra of 2, 4-Dinitrophenylhydrazone of Pyruvic Acid.

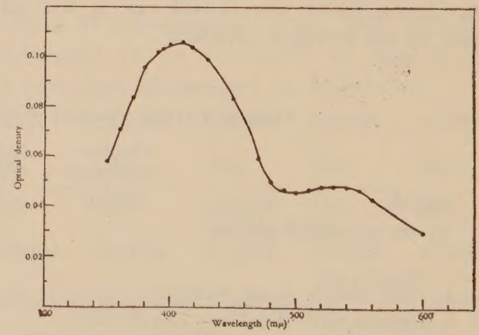


FIG. 2 Absorption Spectra of 2, 4-Dinitrophenylhydrazone of α-Ketoglutaric Acid.

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[17] O. Cavalini, N. Frontari and G. Toschi, *Nature*, **163**, 568 (1949).

[18] L.B. Lockwood and F.H. Stodola, *J. Biol. Chem.*, **164**, 81 (1946).

α-ketoglutaric acid and suggested that α-ketoglutaric acid might be a sort of product of oxidative fermentation. Subsequently, the following experiments were performed by shaking culture which is more aerobic compared with surface culture. The composition of the cultural medium was similar to the previous experiment of static culture except the sources of carbon and nitrogen. *E. coli*, strain No. G–2 was inoculated to 500 ml flasks containing 100 ml of the medium and placed on a shaker of 100 r.p.m.. After incubation at 28°C, for 5 days, the fermentation products were analysed. The results are shown in Table III.

TABLE III

FERMENTATION PRODUCTS BY SHAKING CULTURE

Glucose used (%)	2.0	4.8
$(NH_4)_2HPO_4$ used (%)	0.05	0.1
$(NH_4)_2SO_4$ used (%)	0	0.1
Glucose consumed (%)	100.0	100.0
Lactic acid formed (%)	0	0
Acetic acid formed (%)	0	0
α-Ketoglutaric acid formed (%)	40.0	45.0
Pyruvic acid formed (%)	0	0

As it will be seen in Tables I, II and III, remarkable changes took place in the ultimate products of fermentation, when *E. coli* was cultivated under aerobic conditions by the replacement of conventional anaerobic cultivation. Namely, *E. coli* haas been found to produce a large quantity of α-ketoglutaric acid in place of lactic acid, as the main fermentation product under aerobic conditions. In other words, it is obvious that the lactic acid fermentation is converted to α-ketoglutaric acid fermentation under aerobic conditions such as shaking culture.

Isolation and Identification of α-Ketoglutaric Acid from the Shaking Culture

Separation of Calcium α-Ketoglutarate. A portion of the fermentation liquor was filtered through the pulp of filter paper in order to remove bacterial cells and residual $CaCO_3$. The filtrate was treated with decolorizing carbon at 50°C and the transparent liquor was concentrated to a small volume in vacuo at 35°C. After being kept overnight, fine needle crystals of calcium α-ketoglutarate were obtained, which were difficultly soluble in water.

Isolation of Free α-Ketoglutaric Acid. The calcium salt of α-ketoglutaric acid was suspended in warm water, acidified by HCl and extracted with ether. Crystals of α-ketoglutaric acid were obtained from the ether extract. After recrystallization, a crystal which melted at 115–116°C was obtained. This crystal was verified by mixed melting point test with synthetic α-ketoglutaric acid.

Identification of 2,4-Dinitrophenylhydrazone of α-Ketoglutaric Acid. Moreover, α-ketoglutaric acid was isolated as 2, 4-dinitrophenylhydrazone from the calcium salt. In the first place, the purity of calcium α-ketoglutarate obtained by the shaking culture was estimated from the weight of 2, 4-dinitrophenylhydrazone by the method of Lockwood et al.[18] To 50 mg of the calcium salt, 2.5 ml of 2 N–HCl in which 60 mg of 2,4-dinitrophenylhydrazine was dissolved on the steam bath were added. After being kept for 1 hour at room temperature, the mixture was filtered through a Gooch crucible. The precipitate formed was washed with 3 ml of 2 N–HCl and then 3 ml of water. The crucible was dried at 110°C for 1 hour and the amount of α-ketoglutaric acid was calculated from the weight of the hydrazone. According to this method of analysis, the purity of the calcium salt of α-ketoglutaric acid was found to be 95% of the theoretical value. In the next place, recrystallization of 2, 4-dinitrophenylhydrazone was performed as follows: the 2, 4-dinitrophenylhydrazone was dissolved in ethylacetate and then extracted with 10% Na_2CO_3 solution. When the carbonate extract was acidified by conc. HCl, a pale yellow crystalline precipitate was formed, and the precipitates were then

TABLE IV

OXIDATION PRODUCTS WITH VARIOUS SPECIES OF *COLI-AEROGENES* TYPES

Species of *coli-aerogenes* type	*E. coli neapolitana*	*E. coli communior*	*Bact. friedlander*	*B. acidi lactici*	*Aerobacter aerogenes* B–1	*Aerobacter aerogenes* B–2
Glucose consumed (%)	100.0	100.0	100.0	87.3	88.6	90.0
Acetic acid formed (%)	12.8	4.2	0	0	0	10.6
α-Ketoglutaric acid formed (%)	19.7	29.3	44.4	36.7	29.6	24.2
Pyruvic acid formed (%)	0	0	0	0	3.1	2.8
Acetoin formed (%)	0	0	0	0	2.4	3.2

crystallized from ethanol and water. After further recrystallization from ethylacetate, the crystals were dried in a desiccator over $CaCl_2$. Paper chromatography of this crystal showed only one spot corresponding to 2, 4-dinitrophenylhydrazone of α-ketoglutaric acid. The melting point of this crystal was 220°C (decomp.), not depressed by the admixture with an authentic specimen. Results of analysis were as follows:

Found: C, 40.75; H, 3.06

Calcd. for $C_{11}H_{10}O_8N_4$: C, 40.49; H, 3.07

Subsequently, the oxidative fermentation of glucose by shaking culture at 28°C for 5 days, with various species of *coli-aerogenes* types was investigated with the medium containing 2% glucose and 0.05% $(NH_4)_2HPO_4$, the results of the experiments are shown in Table IV.

As it will be seen from Table IV, a number of microorganisms of *coli-aerogenes* types was recognized to accumulate α-ketoglutaric acid as the oxidation product of their fermentation, although remarkable difference was pointed out among the yields of α-ketoglutaric acid, since some species of the bacteria produced none of the metabolic intermediates besides α-ketoglutaric acid, while acetic acid or pyruvic acid and acetoin were produced by the other species of the bacteria in addition to α-ketoglutaric acid.

Simple Method of Estimating α-Ketoglutaric Acid

The photometric[16,19] and polarographic[20] methods which have generally been used for the estimation of α-ketoglutaric acid, are found to be relatively complicated but they are excellent micro-methods for its determination.

The authors have deviced a method, having a great advantage of rapidity and simplicity, which principle is based on the nature of α-ketoglutaric acid forming bisulphite compound at room temperature in the presence of an excess of sodium bisulphite. The bisulphite compound is hydrolysed when the solution is made alkaline with sodium bicarbonate or sodium carbonate; then the liberated bisulphite is determined by titration with a standard iodine solution as in the case of pyruvic acid shown by Clift and Cook[21].

Procedure of the Estimation. A 5-ml aliquot of the fermented liquor (without pyruvic acid) was acidified (pH 2.0) with HCl and then added 1.0–2.0 ml of about 10% solution of sodium bisulphite ($NaHSO_3$). The solution was kept for 20–30 min., then the excess of bisulphite was removed exactly by the addition of $\frac{N}{10}$ iodine solution using starch solution as the indicator. Sodium bicarbonate or sodium carbonate was then added to make the solution alkaline and the liberated bisulphite titrated again with $\frac{N}{10}$ iodine solution.

1 ml of $\frac{N}{10}$ iodine solution = 7.3 mg of α-ketoglutaric aicd.

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Microbiological Studies of *Coli-aerogenes* Bacteria

Part II. Oxidative Fermentation of Glucose

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Experiments on the oxidative fermentation of glucose were carried out with a number of *coli-aerogenes* bacteria, and remarkable differences were pointed out among the yields of their fermentation products.

The types of fermentation observed by shaking cultures of the bacteria were described as follows : α-ketoglutaric acid-, pyruvic acid-, acetic (pyruvic) and α-ketoglutaric acids- fermentations, and the complete oxidation to CO_2 and H_2O. α-Ketoglutaric acid was obtained in a good yield (50–40%), from glucose in case of α-ketoglutaric acid fermentation.

INTRODUCTION

In their previous paper[1], the authors found that a large amount of α-ketoglutaric acid was produced from glucose by the shaking culture of the bacteria of *coli-aerogenes* type, in other words, lactic acid fermentation revealed by the bacteria was converted to α-ketoglutaric acid fermentation by molecular oxygen.

Among their investigations on bacterial oxidation of glucose which was at first, changed to gluconic acid and then into 2-ketogluconic acid, Lockwood and Stodola (1946)[2] found that one species of *Pseudomonas fluorescens* produced a considerable amount of α-ketoglutaric acid from glucose under aerobic conditions. The production of α-ketoglutaric acid by *Pseudomonas* bacteria was also ascertained by Koepsell[3], Koepsell, Stodola and Sharpe[4], Masuo et al.[5], Asai, Aida and Sugisaki[6]. The production of α-ketoglutaric acid with other microorganisms has been also reported by Dagley, Fewster and Happold[7] with *Vibrio ;* by Hockenhull, Wilkin and Winder[8] with *Penicillium chrysogenum ;* by Walker, Hall and Hapton[9] with *Aspergillus niger ;* by Hunter[10] with *Mycobacterium butyricum ;* by Sakaguchi, Takahashi et el.[11] with *Aspergillus oryzae* and *Rhizopus ;* by Katagiri and Tachibana[12] with a mutant of *Aspergillus oryzae ;* by Asai and Aida et al. with *Serratia marsescens*[5,13,14], *B. megatherium*[15], *B. natto*[15], *Gluconoacetobacter cerinum*[16] and

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Bacterium strain 840[17]; by Masuo, Wakisaka and Kondo with *Bacterium ketoglutaricum*[18] and the genus *Streptomyces*[19] and by Chiretti and Barron with *Corynebacterium creatinovorans*[20]. In the present paper, experiments were carried out with a number of *coli-aerogenes* bacteria in order to ascertain how many kinds of products would appear and how many types of fermentation would exist under aerobic conditions.

EXPERIMENTAL AND RESULTS

Analytical procedure was performed by the methods mentioned in the previous paper.

Production of Acetic, Pyruvic and α-Ketoglutaric Acids by the Shaking Cultures of Various Strains of *Coli-aerogenes* Types

As was previously reported by us, the members of the bacteria belong to the genus *Escherichia* and the genus *Aerobacter* which are gram negative and rod-shaped cells without endospores are found to accumulate a large amount of α-ketoglutaric acid in their cultural solutions. In the following experiments, the production of acetic, pyruvic and α-ketoglutaric acids from glucose was investigated with the other species of *coli-aerogenes* types.

The bacteria were grown in the media containing 2.0–10% glucose, 0.05–0.15% $(NH_4)_2HPO_4$, 0.15% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.5% NaCl and 0.05% $MgSO_4 \cdot 7H_2O$ in distilled water to which sterilized $CaCO_3$, corresponding to 75% of glucose used was added at the time of inoculation. The medium (100 ml) was taken in a 500-ml flask and sterilized. After inoculation with 0.5–2 ml of 20–40 hours' culture of the bacteria on bouillon, the flask was placed on the shaker at the rate of 100–130 r.p.m.. After incubation, fermentation products were analysed. Table I, II and III show the results of the experiments. As it will be seen in Tables, I, II and III a number of the bacteria of *coli-aerogenes* types produced α-ketoglutaric acid by shaking culture as it was verified in the previous paper.

TABLE I

PRODUCTION OF α-KETOGLUTARIC ACID BY VARIOUS SPECIES OF *AEROBACTER*

Strain No.	Consumed Glucose (g/dl)	α-Ketoglutaric acid Produced (g/dl)
B–C	9.000	1.642
B–A	8.000	2.182
B–B	8.780	1.792
B–D	8.000	1.641

Cultivated 4 days at 30°C on the medium containing 10% glucose, 0.15% $(NH_4)_2HPO_4$, and 0.15% $(NH_4)_2SO_4$.

TABLE II

PRODUCTION OF α-KETOGLUTARIC ACID BY VARIOUS SPECIES OF *E. COLI*

E. Coli (Strain No.)	Consumption of Glucose (%)	α-Ketoglutaric Acid Produced (mg–dl)
1	95.0	525.0
3	94.9	600.0
5	88.0	575.0
6	88.0	773.8
8	64.6	437.5

Cultivated 7 days at 30°C on the medium containing 2.0% glucose and 0.05% $(NH_4)_2HPO_4$.

TABLE III

PRODUCTION OF ACETIC, PYRUVIC AND α-KETOGLUTARIC ACIDS BY THE STRAINS OF *COLI-AEROGENES* TYPES

Organisms	*E. coli*			*Aerobacter* (*Bact. succinicum*)
	25 E	26 S	2 E	
Glucose consumed (%)	100.0	80.0	71.6	90.0
Acetic Acid produced (% on consumed glucose)	13.0	31.2		14.6
Pyruvic acid produced (% on consumed glucose)	0	6.6	16.6	41.4
α-Ketoglutaric acid produced (% on consumed glucose)	20.0	9.5	19.5	7.5

Cultivated 6 days at 30°C on the medium containing 2.0% glucose and 0.05% $(NH_4)_2HPO_4$.

Recently Asai, Aida and Sugisaki[15] reported that *Bact. succinicum* produced a considerable amount of α-ketoglutaric acid and a small quantity of pyruvic acid in the course of oxidation of glucose, the maximum yields of α-ketoglutaric and pyruvic acids were 13.6% and 2.5% on glucose used, respectively.

As it will be seen in Table III, a high yield (41.4% on glucose consumed) of pyruvic acid was obtained by *Bact. succinicum* which belonged to the genus *Aerobacter* and was isolated by Sakaguchi and Tada[21] in 1938. In other words it is apparent that pyruvic acid fermentation occurred by the shaking culture of *Bact. succinicum*.

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[17] T. Asai, K. Aida, Z. Sugisaki and N. Yakeishi, *J. General and Applied Microbiology*, **1**, 308 (1955).

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Furthermore, it will be pointed out in Table III that acetic and α-ketoglutaric acids-fermentation and acetic, pyruvic and α-ketoglutaric acids-fermentation took place by the other strains (25 E and 26 S respectively) of *coli-aerogenes* types, the maximum yield of acetic acid was 31.2% on glucose consumed. These experimental results suggest that α-ketoglutaric acid would be produced from 3-carbon or 2-carbon compounds, such as pyruvic and acetic acids. Koepsell et al.[3,4] investigated the oxidations of gluconate and 2-ketogluconate by one species of *Pseudomonas fluorescens* and suggested that α-ketoglutaric acid would arise during the further oxidation of pyruvic acid. Their assumption was derived from their suggestion by the apparent transformation of pyruvic acid into α-ketoglutaric acid at the later phases of fermentation with *Ps. fluorescens*.

Effect of Nitrogen Source upon Production of α-Ketoglutaric Acid. by *E. coli*

In the following experiments, relations of the kind and the amount of sources of nitrogen to the production of α-ketoglutaric acid were investigated with the media containing 2.0 g glucose, mineral matters and various concentrations and various kinds of nitrogen substances described in Tables IV, V, VI and VII in 100 ml of distilled water.

TABLE IV
INFLUENCE OF $(NH_4)_2HPO_4$ ON THE PRODUCTION OF α-KETOGLUTARIC ACID

Concertain of $(NH_4)_2HPO_4$%	0.05	0.10	0.20	0.30
Glucose consumed (%)	70.0	100.0	100.0	100.0
α-Ketoglutaric acid produced (% on consumed glucose)	40.0	45.0	12.5	trace
Bacterial cells formed (relative value)	1	2	6	7

Cultivated 5 days at 30°C on 2% glucose medium.

TABLE V
INFLUENCE OF PEPTONE ON THE PRODUCTION OF α-KETOGLUTARIC ACID

Concentration of peptone (%)	0.05	0.10	0.50	1.00	2.00
Glucose consumed (%)	50.9	78.2	85.2	90.0	100.0
α-Ketoglutaric acid produced (mg/dl)	528.2	777.2	204.4	152.4	trace

Cultivated 5 days at 28°C on 2% glucose medium.

TABLE VI
INFLUENCE OF YEAST EXTRACT ON THE PRODUCTION OF α-KETOGLUTARIC ACID

Concentration of yeast ext. (%)	0.25	0.50	1.00
Glucose consumed (%)	38.5	62.7	86.7
α-Ketoglutaric acid produced (mg/dl)	357.7	496.4	584.0

Cultivated 4 days at 28°C on 2% glucose medium.

TABLE VII
INFLUENCE OF Na-ASPARTATE AND Na-GLUTAMATE ON THE PRODUCTION OF α-KETOGLUTARIC ACID

Added (%)	Na-Aspartate			Na-Glutamate	
	0.13	0.20	0.40	0.20	0.40
Glucose consumed (%)	90.0	100.0	100.0	100.0	100.0
α-Ketoglutaric acid produced (mg/dl)	858.4	902.8	754.8	1070.4	917.6

Cultivated 6 days at 30°C on 2% glucose medium.

From the results shown in Tables IV, V, VI and VII, it will be recognized that concentrations of the nitrogen substances would be more important for the production of α-ketoglutaric acid than the kinds of nitrogen. At the low levels of nitrogen, the rate of fermentation was slow but α-ketoglutaric acid was obtained in high yields. On the other hand, at the high levels of nitrogen, the rate of fermentation was observed to be much more rapid, and enormous growth of bacteria had taken place but the yields of α-ketoglutaric acid were found to be low or nil. For instance, when 2.0% of glucose was employed as the source of carbon, the optimal concentration of $(NH_4)_2HPO_4$ for the production of α-ketoglutaric acid was observed to be 0.05–0.10% as will be seen in Table IV in which the yield of α-ketoglutaric acid reached the level of 40–45% on consumed glucose.

Effect of Vitamin B_1 and Similar Compounds on Pyruvic and α-Ketoglutaric Acids Fermentation

It was anticipated that vitamin B_1 (I) would reveal a noticeable effect on the decomposition of pyruvic and α-ketoglutaric acids by the bacteria of *coli-aerogenes* types, since vitamin B_1 was already ascertained to be a principal component of coenzymes relating to pyruvic and α-ketoglutaric oxidases by Gunsalus et al.[22,23,24]

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[21] K. Sakaguchi and S. Tada, *J. Agr. Chem. Soc. Japan*, **14**, 374 (1938).

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[23] I.G. Gunsalus, Mechanism of Enzyme Action. Ed. W.D. McElroy, p. 545 (1954).

[24] L.P. Hager, J.D. Forthney and I.C. Gunsalus, *Fed. Proc.*, **12**, 213 (1953).

1) Effect of Vitamin B_1. When pyruvic acid fermentation bacteria such as *Bact. succinicum,* were cultivated aerobically in the presence of vitamin B_1, it can be seen in table VIII in which the bacterium was cultured at 30°C for 4–8 days on the shaker with 2% glucose medium mentioned in the previous experiments that the production of pyruvic acid was diminished remarkably and the formation of acetic acid was very much accelerated while any noticeable effect of vitamin B_1 was never observed on the production of α-ketoglutaric acid.

2) Effect of Thiamine Allyl Disulfide and Thiamine Propyl Disulfide. To the same 2% glucose media, these thiamine compounds were added in place of vitamin B_1, inoculated the same bacterium as mentioned above and cultured aerobically at 30 C for 5 days. A noticeable decrease of pyruvic acid was again resulted by the addition of these thiamine compounds as shown in Tables IX and X.

3) Effect on α-Ketoglutaric Acid Fermentation. The fact that any noticeable effect of vitamin B_1 could not be observed on the production of α-ketoglutaric acid by the bacteria to reveal pyruvic acid fermentation is again ascertained by experiments with the bacterial strain producing α-ketoglutaric acid with high potency, as will be seen in Table XI.

TABLE VIII

EFFECT OF VITAMIN B_1 ON THE PRODUCTION OF PYRUVIC ACID

Exp. No.	1	2	3	4
Addition of B_1 (mg/dl)	0	0.5	1.0	3.0
Glucose consumed (%)	88.3	76.2	77.0	77.0
Acetic acid produced (mg/dl)	263.0	478.4	499.2	499.2
Pyruvic acid produced (mg/dl)	747.0	103.5	103.5	100.8
α-Ketoglutaric acid produced (mg/dl)	136.6	197.3	134.2	197.3

TABLE IX

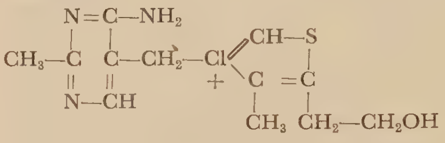
EFFECT OF THIAMINE ALLYL DISULFIDE (ALLITHIAMINE) ON THE PRODUCTION OF PYRUVIC ACID

Addition of allithiamine (γ/dl)	0	15	20
Glucose consumed (%)	80.3	60.0	65.1
Acetic acid produced (mg/dl)	241.0	325.5	347.0
Pyruvic acid produced (mg/dl)	760.5	110.4	135.0
α-Ketoglutaric acid produced (mg/dl)	333.9	288.4	273.2

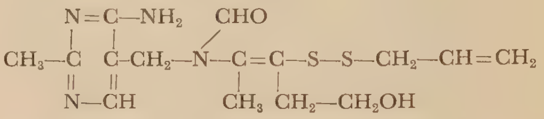
TABLE X

EFFECT OF THIAMINE PROPYL DISULFIDE ON THE PRODUCTION OF PYRUVIC ACID

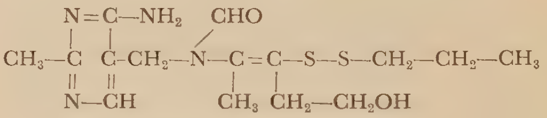
Addition of thiamine propyl disulfide (γ/dl)	0	10	40
Glucose consumed (%)	96.0	85.3	87.9
Acetic acid produced (mg/dl)	174.0	362.0	384.5
Pyruvic acid produced (mg/dl)	477.0	264.0	132.0
α-Ketoglutaric acid produced (mg/dl)	210.0	235.5	300.0



[I] thiamine (vitamin B_1)



[II] thiamine allyl disulfide



[III] thiamine propyl disulfide

TABLE XI

EFFECT OF VITAMIN B_1 AND THIAMINE ALLYL DISULFIDE ON α-KETOGLUTARIC ACID FERMENTATION

Added (γ/dl)	Thiamine allyl disulfide			Vitamin B_1	
	0	80	2000	0	500
Glucose consumed (%)	86.9	90.0	82.0	79.0	78.9
Acetic acid produced (mg/dl)	23.2	25.5	23.2	20.8	20.0
α-Ketoglutaric acid produced (mg/dl)	843.0	810.0	840.0	743.8	774.2

Decomposition of α-Ketoglutaric Acid by *Escherichia Coli* in Growing Culture

In order to investigate the decomposition of α-ketoglutaric acid, one strain of *E. coli* (No. G–2) was incubated in a medium containing 2.0% glucose, 0.05% $(NH_4)_2HPO_4$ and the other mineral matters under aerobic conditions by a shaker at 27°C for 24 hours, and thereafter, the fermentation was allowed to proceed under statical conditions at 30°C, for 25 days. The results of the experiments showed that 30.0% of the glucose used was decomposed and 150 mg of α-ketoglutaric acid was produced from 100 ml of cultural solution by 24 hours' shaking culture, and then 121.0 mg of α-ketoglutaric acid remained while glucose was completely decomposed during 25 days' statical culture. Thus, it will be indicated that α-ketoglutarate, being produced by shaking culture is hardly metabolized on statical conditions even in such long periods.

Furthermore, the influence of the members of tricarboxylic acid cycle on the accumulation of α-ketoglutaric acid in growing culture was investigated as follows: *E. coli*, strain No. G–2, was inoculated on the media added a single acid (Table XII) or mixed acid (Table XIII) composed of a small amount of acetate, succinate and citrate to the basal ingredients mentioned above and cultured at 30°C, for 6 days on a shaker.

As it will be seen from Tables XII and XIII, there was not any noticeable effect of the members of T.C.A. cycle ever to be observed on both the rate of fermentation and production of α-ketoglutaric acid.

Consumption of Glucose and Accumulation of α-Ketoglutaric Acid during Fermentation

In order to trace the mode of oxidation of glucose and the accumulation of α-ketoglutaric acid, one strain of *E. coli* (G–2) giving a high yield of α-ketoglutaric acid was grown on the medium containing 2.0–6.8% glucose, 0.15–0.10% $(NH_4)_2HPO_4$, 0–0.10% $(NH_4)_2SO_4$ and the other mineral matters, under aerobic conditions. The amounts of glucose consumed and of α-ketoglutaric acid produced were estimated with 10 ml of the culture withdrawn at the requisite period of fermentation. From the results shown in Figs. 1 and 2 in which the amounts of residual glucose and α-ketoglutaric acid produced were plotted against the periods of incubation, it will be seen that both the consumption of glucose for growth of the bacterium and the onset of the production of α-ketoglutaric acid were attained within 14 hours, production of α-ketoglutaric there after proceeding rapidly.

The yield of α-ketoglutaric acid was observed to be about 47% on the glucose consumed after 85 hours' incubation when 2.0% glucose was employed, and with 6.8% glucose the yield was found to be about 41% on glucose consumed after 86 hours' incubation. Lockwood et al.[2,4] demonstrated the production of gluconic, 2-ketogluconic, pyruvic and α-ketoglutaric acids from glucose during the growth of *Ps. fluorescens.* Paper chromatography[4] was employed for the detection of various substances such as gluconic, 2-ketogluconic, pyruvic and α-ketoglutaric acids etc., however, none of these metabolic intermediates except α-ketoglutaric acid were ascertained in the growing culture of *E. coli* strain G–2.

TABLE XII

EFFECT OF SUCCINIC AND CITRIC ACIDS ON THE PRODUCTION OF α-KETOGLUTARIC ACID

Added (Mol)	Ca-succinate		Na-citrate		None
	10^{-3}	2×10^{-3}	2×10^{-3}	4×10^{-3}	
Glucose consumed (%)	80.9	83.6	84.7	77.4	80.3
α-Ketoglutaric acid produced (mg/dl)	815.7	814.0	888.0	784.0	754.8

TABLE XIII

EFFECT OF ACETIC, SUCCINIC AND CITRIC ACIDS ON THE PRODUCTION OF α-KETOGLUTARIC ACID

Exp. No.	Conc. of comp. added (M)			Glucose consumed (%)	α-Ketoglutaric acid produced (mg/dl)
	Na-acetate	Ca-succinate	Na-citrate		
1	10^{-3}	10^{-3}	2×10^{-3}	85.2	871.0
2	4×10^{-3}	2×10^{-3}	4×10^{-3}	80.3	722.0
3	None	None	None	76.9	750.0

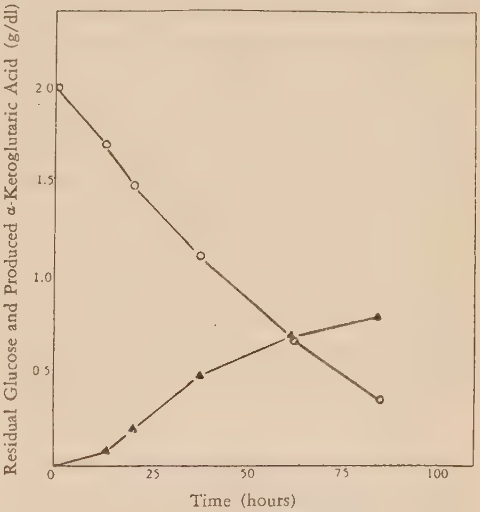


FIG. 1. Consumption of Glucose ○——○ and Production of α-Ketoglutaric Acid ▲——▲ on a Medium Containing 2.0% Glucose and 0.10% $(NH_4)_2HPO_4$.

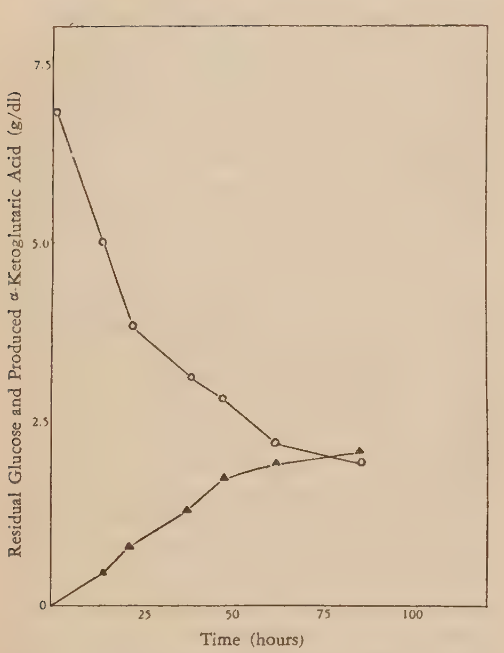


FIG. 2. Consumption of Glucose ○——○ and Production of α-Ketoglutaric Acid ▲——▲ on a Medium Containing 6.8% Glucose, 0.15% $(NH_4)_2HPO_4$ and 0.10% $(NH_4)_2SO_4$

Influence of Concentration of Glucose on Production of α-ketoglutaric Acid

The fermentations of some species of *coli-aerogenes* types at 28–32°C, on a shaker were carried out with the media containing various concentrations (2–9%) of glucose, 0.05–0.15% $(NH_4)_2HPO_4$, 0.05–0.20% $(NH_4)_2SO_4$, $CaCO_3$ corresponding to 75% of glucose used and the other mineral matters, and results of these experiments are shown in Tables XIV and XV. It will be seen in Table XIV that the amount of nitrogen substances had a remarkable effect on the production of α-ketoglutaric acid. Namely, in the case of 4.94% glucose media, when the mixture of 0.15% $(NH_4)_2HPO_4$ and 0.20% $(NH_4)_2SO_4$ was employed as the source of nitrogen, the rate of fermentation was observed to be very rapid but the yield of α-ketoglutaric acid was found low, while a high yield of α-ketoglutaric acid (about 46% on glucose consumed) was obtained after 85 hours' incubation with the mixture of 0.10% $(NH_4)_2HPO_4$ and 0.10% $(NH_4)_2SO_4$. The facts that good yields (about 50–40%) of α-ketoglutaric acid are obtained with such media of simple composition containing 5–9% glucose and some other mineral matters for 45–140 hours' incubation and that α-ketoglutaric acid is readily obtained from the fermented liquor as its calcium salt when concentration of glucose exceeds 4%, will indicate the possibility to industrialize the preparation of α-ketoglutaric acid by fermentation.

TABLE XIV

α-KETOGLUTARIC ACID FERMENTATION ON 4.94% GLUCOSE MEDIUM

Conc. of $(NH_4)_2SO_4$ (%)	0	0.10	0.20
Conc. of $(NH_4)_2HPO_4$ (%)	0.15	0.10	0.15
Glucose consumed (%)	67.9	100.0	100.0
α-Ketoglutaric acid produced (% on consumed glucose)	49.0	46.0	12.1

TABLE XV

INFLUENCE OF THE CONCENTRATION OF GLUCOSE ON THE PRODUCTION OF α-KETOGLUTARIC ACID

Exp. No.	Strain No.	Time of incubation (hrs.)	Initial conc. of glucose (%)	Glucose consumed (g.)	α-Ketoglutaric acid produced (% on consumed glucose)
1	2 C	84	5.0	4.94	42.9
2	2 C	46	5.0	4.50	42.3
3	B 24	60	5.0	4.70	43.6
4	B 25	60	6.0	5.58	39.8
5	B 24	60	5.0	4.70	44.7
6	B 24	120	8.0	7.61	48.8
7	B 24	120	8.0	6.70	45.0
8	B 25	120	8.0	6.48	51.5
9	2 C	140	9.0	8.33	51.0
10	2 C	140	9.0	8.83	45.6
11	2 C	140	9.0	7.01	49.0
12	5 E	115	9.0	9.00	19.5
13	6 E	115	9.0	8.88	32.4
14	7 E	115	9.0	9.00	33.2
15	8 E	115	9.0	8.73	34.4
16	9 E	115	9.0	9.00	41.8

DISCUSSION

In the previous paper, it has already been found by us that the various species of the genus *Escherichia* and the genus *Aerobacter* reveal their ability of producing a large amount of α-ketoglutaric acid from glucose, when they are cultivated under aerobic conditions such as shaking culture. From the results of the present experiments, it is observed that some strains of the bacteria of *coli-aerogenes* types accumulated none of the metabolic intermediates except α-ketoglutaric acid, while so many products such as acetic, pyruvic and α-ketoglutaric acids were obtaind with other strains of the same types of the bacteria. In other words, so many forms of oxidative fermentation of glucose exist in the metabolisms of the bacteria of *coli-aerogenes* types, as given below:

1) α-Ketoglutaric acid fermentation,
2) Acetic, pyruvic and α-ketoglutaric acids fermentation,
3) Acetic and α-ketoglutaric acids fermentation,
4) Pyruvic acid fermentation,
5) Complete oxidation to CO_2 and H_2O.

It is worth to note that a lower yield of α-ketoglutaric acid was generally observed, when the production of acetic acid was increasing. Lockwood et al. found 2-ketogluconic acid, which in the early stage of fermentation was produced from glucose and that 2-ketogluconic acid was then changed into α-ketoglutaric acid by *Ps. fluorescens.* As for *Serratia marsescens*, similar results were obtained by Asai and Aida et al.[6,13,14] On the contrary, it was demonstrated by us, that 2-ketogluconic acid could not be an intermediate product of α-ketoglutaric acid with *Escherichia coli*, since, 2-ketogluconic acid was never detected at any stage of fermentation. It was also found by us that a remarkable amount of α-ketoglutaric acid was already produced in the early stage of fermentation (within 14 hours' culture) by some species of *coli-aerogenes* types.

Phosphoric acid ester of vitamin B_1 has already been pointed out to be the principal component of the coenzyme of the oxidizing systems of pyruvic and α-ketoglutaric acids by Gunsalus et al. Therefore, they concluded that vitamin B_1 would reveal such a noticeable effect on the decomposition of α-ketoglutaric acid as it had been already ascertained on the decomposition of pyruvic acid. In our experiments with the bacteria of *coli-aerogenes* types, vitamin B_1 was found to diminish the formation of pyruvic acid, however no effect of vitamin B_1 was observed on the decomposition of α-ketoglutaric acid throughout the fermentation.

Acknowledgment The authors are grateful to Dr. R. Kimura of Kyoto University, Dr. K. Sakaguchi and Dr. K. Arima of Tokyo University for kindly supplying the bacterial strains used in this work. This work was supported in part by a Research Grant from the Ministry of Education, to which the author's thanks are due.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 4, p. 222~224, 1957]

Studies on Synthetic Pyrethroid

Part XI.* Another Evidence for the *trans*-Configuration of αδ-Dimethylsorbic Acid

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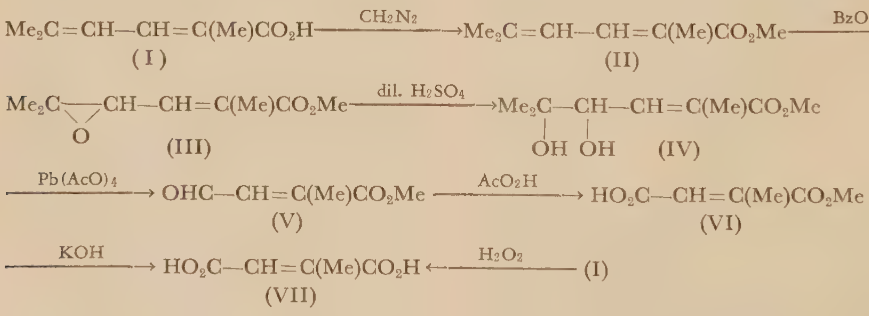
Methyl αδ-dimethylsorbate, when submitted to the selective oxidation sequence by the actions of perbenzoic acid, mineral acid, lead tetraacetate and peracetic acid in this order, finally gives β-monomethyl mesaconate, which is converted after hydrolysis to mesaconic acid of the well-defined *trans*-configuration. The reaction sequence involves no process likely to invert the configuration, and thus another cogent chemical evidence for the *trans*-configuration of αδ-dimethylsorbic acid is obtained in agreement with the physico-chemical conclusion, previously reported.

The assignment of the *trans*-configuration to αδ-dimethylsorbic acid has been provided by physico-chemical processes in our previous paper[1], in which the selective semi-hydrogenation of this acid and ultra-violet, infrared absorption spectra and acid dissociation constant of the hydrogenated product were dealt.

Another cogent chemical evidence for this configuration is presented in this paper.

Heinänen[2], reported that the selective epoxydation occurred at the γδ-double bond of methyl sorbate by the action of perbenzoic acid in the cold, giving methyl γδ-epoxy-Δα-hexenoate.

Epoxydation of methyl αδ-dimethylsorbate (II) by perbenzoic acid gave methyl γδ-epoxy-αδ-dimethyl-Δα-hexenoate (III) as was expected, one atom of oxygen being taken up. The γδ-epoxy structure of III, can reasonably be deduced from the subsequent processes. Conversion of the epoxy-ester into the dihydroxy-compound was easily effected by treatment with dilute sulphuric acid, yielding γδ-dihydroxy-αδ-dimethyl-Δα-hexenoate (IV).



$$\underset{\text{(I)}}{\mathrm{Me_2C{=}CH{-}CH{=}C(Me)CO_2H}} \xrightarrow{\mathrm{CH_2N_2}} \underset{\text{(II)}}{\mathrm{Me_2C{=}CH{-}CH{=}C(Me)CO_2Me}} \xrightarrow{\mathrm{BzO_2H}}$$

$$\underset{\text{(III)}}{\mathrm{Me_2\overset{}{C}\underset{O}{\diagdown\!\diagup}CH{-}CH{=}C(Me)CO_2Me}} \xrightarrow{\text{dil. }\mathrm{H_2SO_4}} \underset{\text{(IV)}}{\mathrm{Me_2\underset{OH}{C}{-}\underset{OH}{CH}{-}CH{=}C(Me)CO_2Me}}$$

$$\xrightarrow{\mathrm{Pb(AcO)_4}} \underset{\text{(V)}}{\mathrm{OHC{-}CH{=}C(Me)CO_2Me}} \xrightarrow{\mathrm{AcO_2H}} \underset{\text{(VI)}}{\mathrm{HO_2C{-}CH{=}C(Me)CO_2Me}}$$

$$\xrightarrow{\mathrm{KOH}} \underset{\text{(VII)}}{\mathrm{HO_2C{-}CH{=}C(Me)CO_2H}} \xleftarrow{\mathrm{H_2O_2}} \text{(I)}$$

Lead tetraacetate cleaved the glycolic carbon-carbon linkage of IV and gave β-monomethyl mesaconaldehydate (V), which was characterized by 2, 4-dinitrophenylhydrazone m.p. 204–4.5°.

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*Part X. *Botyu-Kagaku*, **21**, 105 (1956).

[1] Y. Inouye, T. Sugita and M. Ohno, This Bulletin, **21**, 5 (1957): *Botyu-Kagaku;* **21**, 86 (1956).

[2] P. Heinänen, *Suomen Kemistilehti*, **11**, B. 2 (1938): *Chem. Zent.*, **1938**, 4032

β-Monomethyl mesaconaldehydate was then oxidized with peracetic acid to β-monomethyl mesaconate (VI) m.p. 82–3°, which, as well as the amide m.p. 117° derived therefrom, was completely consistent with the literature[3].

Cold saponification of β-monomethyl mesaconate gave mesaconic acid (VII) m.p. 202–3°, not depressed by the mixed melting point comparison with an authentic specimen.

Since the oxidation sequence involves no process likely to invert the geometrical configuration of the double bond, the retention of the configuration in the parent compound can reasonably be concluded. Thus, the final formation of mesaconic acid of the well-established *trans*-configuration unambiguously shows the *trans*-configuration of the αβ-double bond of the parent methyl αδ-dimethylsorbate.

Direct peroxidation of αδ-dimethylsorbic acid also gave mesaconic acid in an inferior yield.

The chemical evidence mentioned above is in agreement with the physico-chemical conclusion described in our previous paper.

EXPERIMENTAL

All melting and boiling points were uncorrected. Microanalyses were by the Microanalytical Division of Mitsui Laboratory, Kyoto University. Before analyses, samples were completely dried over P_2O_5 *in vacuo* at an appropriate temperature for each.

Methyl αδ-dimethylsorbate (II) To a 4.5 g quantity of αδ-dimethylsorbic acid[4] (I, m.p. 134–5°, 0.032 mole.) dissolved in 200 ml of dry ether and chilled at −15°, were added dropwise 1.3 g of diazomethane in ether (0.032 mole.) under an effective stirring and chilling. The reaction mixture was washed with saturated sodium bicarbonate solution and dried over anhydrous sodium sulphate. After removal of ether, the residue was distilled under a reduced pressure to give methyl αδ-dimethylsorbate*. b.p. 85–6°/7 mm., n_D^{10} 1.5242, yield 4.1 g (85%).

Methyl γδ-epoxy-αδ-dimethyl-Δα-hexenoate (III) Four grams of methyl αδ-dimethylsorbate, (0.027 mole) were dissolved in 5 ml of dry chloroform and to this were added 147 ml of 3.5% perbenzoic acid in chloroform (0.038 mole). The mixture was kept at 0° and the consumption of the active oxygen was estimated by means of iodometry on a small portion drawn from the reaction mixture at intervals. After 5 days' standing, 0.03 atom of oxygen was taken up, then the excess of perbenzoic acid was decomposed with sodium sulphite, removed by washing with sodium carbonate and dried over anhydrous sodium sulphate. After removal of the solvent, the residue was distilled under a reduced pressure to give methyl γδ-epoxy-αδ-dimethyl-Δα-hexenoate, b.p. 91–2°/6 mm., n_D^{20} 1.4672, yield 3.7 g (84%).

Methyl γδ-dihydroxy-αδ-dimethyl-Δα-hexenoate (IV) To a 1.5 g quantity of epoxy-ester (III), was added 0.5 ml of 5% sulphuric acid, kept at room temperature and after several hour it turned homogeneous. This was then extended in 100 ml of alcohol-free ether and was completely dried over anhydrous sodium sulphate. Removal of ether gave methyl γδ-dihydroxy-αδ-dimethyl-Δα-hexenoate in a quantitative yield. plates, (from methanol and benzene) m.p. 50–1° (*Anal.* Found: C, 57.40; H, 8.46, Calcd. for $C_9H_{16}O_4$: C, 57.43; H, 8.57).

β-Monomethyl mesaconaldehydate (V) The dihyroxy-ester (IV, 1.48 g., 0.0079 mole) was dissolved in 100 ml of dry benzene and to this were added 3.9 g of freshly prepared lead tetraacetate (0.0087 mole) in three portions. The mixture was stirred at 50° for 2 hrs. and then at 60° for 2 hrs. After the duration, excess of lead tetraacetate was decomposed with water and lead oxide formed was removed by filtration. The filtrate was dried over anhydrous sodium sulphate and the solvent was distilled off. Distillation of the residue *in vacuo* gave β-monomethyl mesaconaldehydate, b.p. 76–8°/12 mm., n_D^{20} 1.4680 (0.75 g., 75%). *2, 4-Dinitrophenylhydrazone,* orange needles (from methanol), m.p. 204–4.5° (decomp.) (*Anal.* Found: C, 46.73; H, 3.92; N, 18.21, Calcd. for $C_{12}H_{12}O_6N_4$: C, 46.76; H, 3.92; N, 18.18).

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[3] R. Anschütz, *Ann*, **353**, 178 (1907).

[4] Y. Inouye et al, This Bulletin, **19**, 193 (1955): *Botyu-Kagaku*, **20**, 102 (1955).

*This ester is completely identical with that obtained by the dehydration of the corresponding Reformatski ester.[4] This reconversion of the free acid into the original ester excludes the stereomutation during the hydrolysis and provides further certification for the homogeneity of the *trans*-ester used in our total synthesis of chrysanthemum dicarboxylic acid.

β-Monomethyl mesaconate (VI) The aldehyde-ester (V, 0.3 g., 0.0024 mole.) was dissolved in 2.7 ml of 13.3% peracetic acid (0.0047 mole) and the mixture was kept cold for 36 hrs.. Then the mixture was diluted with water and dried up *in vacuo* to yield a crystalline mass. The residue was crystallized from petroleum ether (b.p. 40–50°) to give β-monomethyl mesaconate (0.31 g), needles, m.p. 82–3° (*Anal.* Found C, 50.04, H, 5.61, Calcd. for $C_6H_8O_4$, C, 50.00, H, 5.60) *Amide*, needles (from ether-petroleum ether), m.p. 115.5–6.5° (*Anal.* Found : C, 50.39 ; H, 6.51 ; N, 9.72, Calcd. for $C_6H_9O_3N$: C, 50.34 ; H, 6.34 , N, 9.79). These were perfectly consistent with the literature[3].

Mesaconic acid (VII) β-Monomethyl mesaconate (VI, 0.1 g) was hydrolysed with cold 5% ethanolic potassium hydroxide, dried up *in vacuo* and acidified with dilute sulphuric acid. This was extended in ether, dried over anhydrous sodium sulphate and the removal of ether gave mesaconic acid, cubes (from water) m.p. 202–3° (*Anal.* Found : C, 46.07 ; H, 4.60, Calcd. for $C_5H_6O_4$: C, 46.16 ; H, 4.65). The melting point was not depressed by the mixed melting point comparison with an authentic specimen prepared by recorded method[5]. *bis-p-Phenylphenacylester,* fine needles (from chloroform), m.p. 204–5° (*Anal.* Found : C, 76.35 ; H, 5.14, Calcd. for $C_{33}H_{26}O_6$: C, 76.43 ; H, 5.05).

Peroxidation of αδ-dimethylsorbic acid (I) αδ-Dimethylsorbic acid (0.5 g) was dissolved in 10 ml of dilute acetic acid, and to this were added 5 ml of 30% hydrogen peroxide. The mixture was warmed on a water bath for about 10 hrs. with additional peroxide at intervals. At the end of this time, the solution was dried up *in vacuo* and the solid residue was extracted with cold ether. To the remaining residue were added a drop of dilute sulphuric acid and 2 ml of water, and it was warmed on a water bath. Reduction of the volume gave mesaconic acid (19 mg), which melted at 201–3° after recrystallization from water. The melting point was not depressed by admixture with an authentic specimen. Further reduction of the filtrate gave a less pure product (ca. 25 mg).

The authors are indebted to Prof. S. Takei for his interest in this work and to Ass. Prof. M. Nakajima for his valuable advice. This work was supported in part by a Research Grant from the Ministry of Education.

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[5] *Org. Synth.*, Coll. Vol. **II**, 382.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 4, p. 225~229, 1957]

Studies on the Conjugated Lipids

Part XI. Hydrolysis of Cerebroside Sulfate by Shellfish Liver Enzyme

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The enzyme, which hydrolyzes the sulfuric ester linkage in the molecule of cerebroside sulfate, was investigated. Among the shellfish liver enzyme preparations examined, it was found that the abalone liver possessed a sulfatase activity that liberates inorganic sulfate from the compound, having no activity to split the galactosidic and peptidic bond of the molecule. The same enzyme preparation was also observed to hydrolyze chondroitin sulfate but not phenol sulfate and glucose sulfate at all.

Cerebroside sulfate is a sulfur-containing lipid, which was first isolated from the beef brain by Blix[1] in 1933, and its chemical structure was well established recently by Thannhauser and his co-workers[2], as shown in Fig. 1. The cerebroside sulfate is composed of each one molecule of sphingosine, cerebronic acid, galactose and sulfuric acid as the constituents, corresponding to a sulfuric ester of phrenosine. Since both cerebroside sulfate and phrenosine are distributed in the common tissues, e. g., in the mammalian brain, and are analogus to each other in respect of the chemical structure, it is not unreasonable to presume that there may exist a close correlation between the two compounds in the metabolic system of animals. Phrenosine might for instance be produced in part through an enzymatic hydrolysis of cerebroside sulfate in vivo, and, if so, some kinds of sulfatase must participate in the hydrolytic reaction.

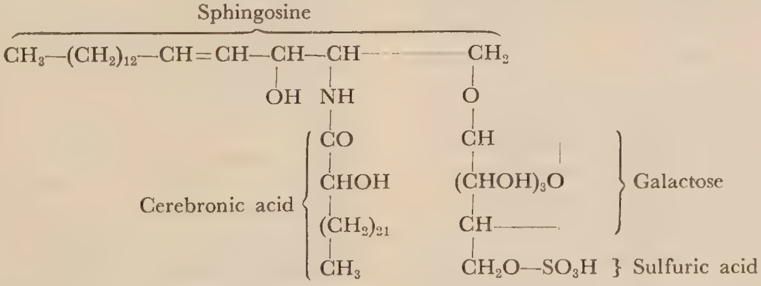

FIG. 1 Structure of Cerebroside Sulfate

The sulfatase are enzymes which hydrolyze various sulfuric esters to produce alcohol and sulfuric acid, and the following four types of sulfatases have been classified with respect to the nature of the substrates which they hydrolyze: phenolsulfatase, glucosulfatase, chondrosulfatase and myrosulfatase. These sulfatases are known to occur in mammalian tissues, molluscs, microorganisms and some kinds of plants. The present authors at first attempted to hydrolyze cerebroside sulfate by several enzyme preparations originating from mammals or microorganisms, but results were negative so far as the enzyme preparations tested are concerned. Investigation was next directed to the field of molluscs, and, it was finally detected that the enzyme preparation from the abalone liver had a sulfatase activity toward cerebroside sulfate among the preparations from several shellfish livers, though the degree of hydrolysis was not great.

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[1] A. Blix, *Z. physiol. Chem.*, **219**, 82 (1933).

[2] S.J. Thannhauser, J. Fellig and G. Schmidt, *J. Biol. Chem.*, **215**, 211 (1955).

The abalone liver enzyme preparations showed no activities for the splitting of the peptidic and galactosidic bond in the molecule of cerebroside sulfate, suggesting that only sulfatase activity was responsible for the first stage of hydrolysis of cerebroside sulfate in this enzyme preparation. Furthermore, when the abalone liver enzyme preparation was tested for hydrolysis of a few representative organic sulfates, namely, phenol sulfate, glucose sulfate and chondroitin sulfate, only the latter compound was to some extent hydrolyzed, whereas, the former two were not at all. It was assumed, therefore, that the cerebroside sulfatase in the abalone liver enzyme preparation might attack the sulfuric ester linkage of condroitin sulfate as well.

EXPERIMENTAL

1. Isolation of Cerebroside Sulfate from Beef Brain. Two procedures have been reported for the isolation of celebroside sulfate, one by Blix1) and the other by Thannhauser and his coworkers2). The present authors first isolated the sphingolipids from the beef brain in accordance with the procedure described in the previous communication3), proceeding to prepare cerebroside sulfate from the sphingolipids, mostly in accordance with the procedure of Blix1). The actual procedures employed are summarized in Fig. 2.

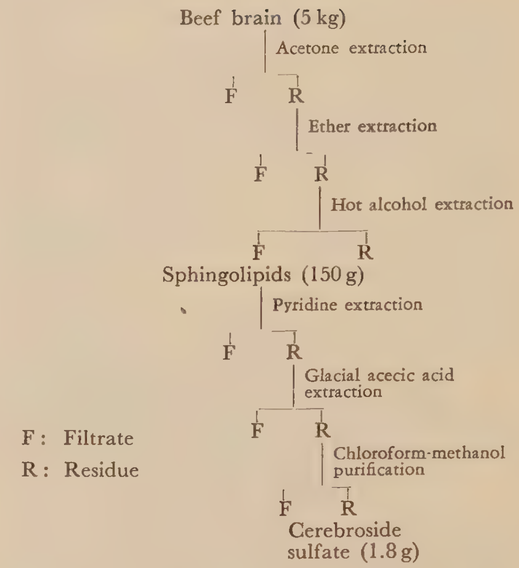

FIG. 2 Isolation of Cerebroside Sulfate

The acetone-ether insoluble material from 5 kg of beef brain was extracted with hot alcohol to obtain sphingolipids in a yield of about 150 g. The sphingolipids would include not only a large amount of sphingomyelines and cerebrosides, but also a small amount of cerebroside sulfate, gangliosides and other contaminating substances. The removal of the contaminating substances, therefore, was carried out on the sphingolipids in order to take out the desired compound, cerebroside sulfate. The sphingolipids were dissolved in five volumes of pyridine with occasional stirring at 60°, and then cooled overnight in an ice box. The resulting precipitate was filtered off from the mother liquor, washed with acetone and dried. In the course of this treatment, a greater portion of the cerebrosides was removed to the filtrate to give a powder, in a yield of about 80 g. The residue was next dissolved in twenty volumes of glacial acetic acid with stirring at 60°, and then allowed to stand at room temperature. The precipitate formed was separated from the mother liquor and washed with acetone. The same treatment was repeated again using ten volumes of glacial acetic acid. During the two extractions with acetic acid, the sphingomyelines and gangliosides went out to the acetic acid-phase giving rise to about 12.5 g of dry material.

The pale yellowish powder thus obtained, was next treated with twenty volumes of boiled chloroform-methanol mixture (2 : 1) and cooled overnight at −15°. After the resulting precipitate was collected by filtration, dissolved in twenty volumes of the same solvent mixture and allowed to cool to about 20°, the precipitate formed was separated. The same procedure with a chloroform-methanol mixture (2 : 1)

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3) Y. Fujino, *J. Agr. Chem. Soc.* (in Japanese), **30**, 764 (1956).

was repeated again, the contaminating material thus being practically eliminated. The final yield obtained was about 1.8 g of cerebroside sulfate, which was in the form of amorphous powder, soluble in 90% acetone and hot chloroform, slightly soluble in alcohol and chloroform-methanol mixture and insoluble in ether, pyridine and glacial acetic acid. *Analysis.* Found: N, 1.46; S, 3.44; P, trace; N : S=1 : 0.91. Calcd. for $C_{48}H_{93}O_{12}NS$ (M.W. 908.3): N, 1.54; S, 3.53; P, 0; N : S=1 : 1.

2. Preparation of Enzyme Solution from Shellfish Liver. The enzyme solution was prepared from shellfish liver according to the method described by Soda[4]. About 40 g of fresh shellfish liver were crushed as fine as possible in 20–30 ml of acetone added. The crushed shellfish liver in acetone was then transferred to a small wire-basket placed in a beaker containing 60–70 ml of acetone. When the crushed material was agitated in the basket immersed in acetone, the fine powder of the liver passed through a net of the wire-basket and piled up in the bottom of the acetone-containing beaker, while the fibrinous stuff of the liver remained in the basket. Acetone was removed by decantation of the beaker, and the powdery residue was agitated in another 100 ml of acetone newly added. The beaker was decanted to flow acetone out, and the residue was agitated again with 200 ml of acetone so as to make dehydration and removal of fats complete. The powdery liver material in the acetone suspension was filtered by aspiration, and, after the residue was dried in air, a brownish green powder was obtained in a yield of 8–10% of the starting shellfish liver.

In order to prepare the enzyme solution, one part of the shellfish liver powder obtained as described above was added to five parts (by volume) of water, mixed to form a homogeneous suspension and allowed to stand at 25° for two days. After autolysis the suspension was centrifuged for twenty minutes at 3,000 r.p.m., and the supernatant solution was employed as the enzyme solution. In case the supernatant solution was turbid, a centrifugation of 12,000 r.p.m. for twenty minutes was effective for obtaining a clear enzyme solution.

3. Determination of Cerebroside Sulfatase Activity. Enzymatic hydrolysis was carried out in 5.0 ml of the reaction mixture composed of the following solutions: a 0.5% solution of cerebroside sulfate (substrate)—0.5 ml, 20% solution of shellfish liver powder (enzyme)—2.0 ml, 0.5 M solution of acetic acid-sodium acetate (buffer)—2.5 ml and toluene (antiseptic)—one drop. The reaction mixture was stoppered and held at pH 5.2 and 25° for definite hours. After incubation, an aliquot of the mixture was subjected to determination of the inorganic sulfate by the colorimetric method of Tanaka[5]. Two milliliters of the reaction mixture was added to the uranium acetate solution and it was centrifuged to remove the proteins, then the sulfate ion in the supernatant was precipitated as benzidine sulfate, the benzidine of which moiety was reacted with *p*-naphthoquinone sulfonic acid and the resulting red color was estimated colorimetrically in comparison with the standard solution of benzidine chloride (1 ml=0.02 mg S). On the complete hydrolysis of the cerebroside sulfate, 2.0 ml of the reaction mixture should liberate the inorganic sulfate, corresponding to 0.035 mg S theoretically.

The control experiment was performed with the substrate and enzyme solution respectively, in parallel with the main experiment. Neither the existence nor liberation of the inorganic sulfate was observed in either of the solutions even after twenty-four hours incubation. The enzymatic activity was thus indicated in terms of the quantity of sulfur calculated from the inorganic sulfate in 2.0 ml of the reaction mixture after incubation.

4. Hydrolysis of Cerebroside Sulfate by Shellfish Liver Enzyme Preparations. The enzyme solutions were prepared from four kinds of shellfish, namely, oyster, round-clam, scallop and abalone, which were examined for their sulfatase activity toward cerebroside sulfate. As shown in Table I, the last one was observed to have a sulfatase activity, whereas, the others caused almost no liberation of inorganic sulfate. The abalone liver enzyme solution hydrolyzed cerebroside sulfate to a degree of 14.3% in three hours but hydro-

TABLE I

HYDROLYSIS OF CEREBROSIDE SULFATE BY SHELLFISH LIVER ENZYMES

(pH 5.2,25°)

Activity / Enzyme from	Libd. S in 2.0 ml of mixture in 0 hr.	3 hrs.	24 hrs.	Degree of Hydrolysis in three hours
Oyster	0mg	0.001mg	0.001mg	3%
Round-clam	0	0	0.001	0%
Scallop	0	0	0.001	0%
Abalone	0	0.005	0.005	14.3%

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[4] T. Soda, from the *Textbook of Enzymological Methods* (in Japanese), **II**, 73–82 (S. Akabori, Ed., The Asakura Press, Tokyo, 823 pp., 1956).

[5] S. Tanaka, *J. Biochem.*, (Japan), **28**, 119 (1938).

lysis did not proceed further even after twenty-four hours. Since the enzymatic hydrolysis was not so active as had been expected, usual activators for sulfatases such as ammonium chloride or magnesium chloride were added to test the activating effect, but the result was negative.

5. Specificity of Action of Abalone Liver Enzyme Preparation. An attempt was made to investigate whether the abalone liver enzyme could hydrolyze the galactosidic linkage between the galactose and sphingosine moiety in the molecule of cerebroside sulfate. For this purpose, the following reaction mixture was incubated at pH 3.6 and 36° for four hours: a 0.5% solution of cerebroside sulfate—2.0 ml, enzyme solution of abalone liver—2.0 ml, 0.01 M solution of methionine — 0.2 ml and buffer solution of glycine-hydrochloric acid—0.8 ml. The galactosidase activity of the enzyme in the mixture was determined according to the procedure reported in the previous communication⁶⁾. One ml of the reaction mixture should liberate 0.4 mg of galactose, if hydrolysis were complete. As a result, no increase of reducing power, by galactose in this case, was observed in the reaction mixture, which means that the abalone liver had no activity of galactosidase to attack the galactosidic linkage of cerebroside sulfate.

Examination was next conducted on the possibility that abalone liver enzyme contained an enzymatic activity which could split down the bond between the cerebronyl group and sphingosine moiety of the molecule. To this aim, the following solutions were mixed and incubated at pH 7.0 and 36° for four hours: a 0.5% solution of cerebroside sulfate—1.8 ml, enzyme solution of abalone liver—2.2 ml and M/15 buffer solution of phosphate—1.0 ml. Fatty acid liberated was determined after incubation according to the Fairbairn titration method⁷⁾. In complete hydrolysis of the substrate, 5.0 ml of the reaction mixture should liberate fatty acid corresponding to 1.0 ml of 0.01 N sodium hydroxide. As a result of the present experiment, no increase of titrable acid was found in the reaction mixture, suggesting that the abalone liver had no enzymatic activity to attack the peptidic bond of the cerebroside sulfate.

These results lead us to the conclusion that the abalone liver had only sulfatase activity and no galactosidase or peptide-splitting activity toward cerebroside sulfate.

6. Hydrolysis of Other Organic Sulfates by Abalone Liver Enzyme Preparation. Since shellfish liver is generally known to possess phenol-, gluco-, chondro- and myro-sulfatase, examination was carried out to confirm whether the abalone liver enzyme preparation contained other sulfatases in addition to the cerebroside sulfatase. Phenol sulfate, glucose sulfate and chondroitin sulfate were selected as representative sulfuric substrates for the present experiment. Potassium phenol sulfate and barium glucose sulfate prepared by the method of Soda⁴⁾, were obtained in a purity of 94% and 88% respectively with respect to the sulfur content of the preparations. *Analysis* of pottasium phenol sulfate. Found: S, 14.2. Calcd. for $C_6H_5O_4KS$ (M.W. 212.3): S, 15.10. *Analysis* of barium glucose sulfate. Found: S, 4.29. Calcd. for $(C_6H_{11}O_9S)_2Ba$ (M.W. 655.8): S, 4.88. Chondroitin sulfuric acid* was extracted from a costal cartilage of calf and it precipitated with alcohol giving a beautiful white powder by the method of Sawjalow⁸⁾. *Analysis.* Found: N, 2.90; S, 6.55. Calcd. for $(C_{14}H_{22}O_{15}NS)_n$ (M.W 476.4×n): N, 2.94; S, 6.72.

Toward these three sulfuric compounds, the sulfatase activity of the abalone liver was examined under the same conditions as those under which the cerebroside sulfatase acted. The composition of the reaction mixture is shown in Table II. The three reaction mixtures were incubated at pH 5.2 and 25° for three hours, and then the liberated inorganic sulfate was determined in each aliquot of the mixture. As shown in Table III, phenol sulfate and glucose sulfate could not be

TABLE II
REACTION MIXTURE FOR THE THREE SULFATASES

Component	Reaction mixture for		
	Phenol-sulfatase	Gluco-sulfatase	Chondro-sulfatase
0.5% Phenol sulfate	0.1 ml	— ml	— ml
0.5% Glucose sulfate	—	0.2	—
0.5% Chondroitin sulfate	—	—	0.3
20% Abalone enzyme	2.0	2.0	2.0
2 N Ba-acetate (pH 5.2)	1.0	1.0	1.0
Distd. water	1.9	1.8	1.7
Sum	5.0	5.0	5.0

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6) Y. Fujino and T. Negishi, This Bulletin **20**, 183 (1956).

7) D. Fairbairn, *J. Biol. Chem.*, **157**, 633 (1945).

* The authors are indebted to Professor H. Masamune, Department of Medical Chemistry, Faculty of Medicine, University of Tohoku, for the pure sample of chondroitin sulfuric acid which was requested for the identification of their preparation.

8) W. Sawjalow, *Z. physiol. Chem.*, **126**, 219 (1923).

TABLE III
ENZYMATIC ACTIVITY OF ABALONE ENZYME TOWARD THREE SULFURIC SUBSTRATES
(pH 5.2, 25°, 3 hrs)

	Activity toward		
	Phenol sulfate	Glucose sulfate	Condroitin sulfate
Liberated S mg+	0	0	0.008
Calculated S mg++	0.038	0.048	0.040
Degree of hydrolysis+++	0%	0%	20%

+ Observed amount of S, mg in 2.0 ml of the reaction mixture.
++ Theoretical amount of S, mg in 2.0 ml of the reaction mixture in complete hydrolysis.
+++ Siberated S/Calculated S×100.

hydrolyzed, whereas chondroitin sulfate was attacked in almost the same degree as cerebroside sulfate. This fact, together with the fact that both cerebroside sulfate and chondroitin sulfate possess the galactose-sulfuric acid moiety in their molecules, may possibly suggest that the cerebroside sulfatase in the abalone liver will hydrolyze the chondroitin sulfate as well in the same manner. Further investigations are now in progress to assure this postulation.

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Studies on the Influence of Different Algerian Rock Phosphates when Reinforced with Leguminous Plant Material Like Cow-Pea (*Vigna-Sinensis*) on Carbon Transformations, Exchangeable Calcium and pH of the Alkali Soil

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The importance of phosphates is due to the reason that phosphorous is an essential constituent of plant and animal life; the quantity in the average soil is relatively small, particularly in alkali soil, and there is no atmospheric supply of this element on which plants can grow. It is seldom that crop fails to respond with increased yields when soluble phosphates are applied to soils that have been under cultivation for 25 years or more. Collings[1] has stated as follows:-

"Low crop yields are more often due to the lack of phosphoric acid than due to the lack of any other nutrient. Phosphoric acid has often been called the 'master key' to agriculture. Phosphoric acid appears to be concerned in the production of nucleo-proteids, and it appears that phosphoric acid influences the production of seeds or grain more particularly than does nitrogen or potash".

It is well known that when water-soluble phosphates are added to the soil, a major part is rapidly converted into insoluble form, i.e., fixed by the soils and only a small portion of the added phosphate is taken up by the plants. Soils vary greatly in their capacity to fix phosphates. The organic matter, the pH, the amount of soluble calcium and magnesium, the amount and chemical nature of the clay colloids and the amount of iron and aluminium present in the soils are all important factors affecting the phoshate fixing power of the soils and the nature of the compound formed. According to Russel[2], in neutral and alkaline soils, the dicalcium phosphate appears to react with more calcium, giving perhaps first a compound of the octa-phosphate type $Ca_4H(PO_4)_3$ before reverting to an apatite CaX_2, $Ca_3(PO_4)_2$ where $X=OH$ or F.

The present studies were undertaken by us to see the effect of decomposing organic matter on the increased availability of phosphates added and the beneficial effect of the solubilized phosphates on the pH value of the alkali soil together with an increase in the availability of phosphate and exchangeability of calcium.

METHODS AND MATERIALS

Total carbon was estimated by the Robinson, McLeans and Williams[3] method. Exchangeable calcium was determined by the NaCl method of Hissinik[4]. Available phosphate was determined by the 2% citric acid method of Dyer[5]; the pH was determined by a Beckman pH-meter.

The soil used in the experiments was collected from "Community Project Area", Phulpur Allahabad (India) and from Soraon, Allahabad, (India). Five samples of Algerian rock phosphates were obtained from France. The leguminous plants (cow-pea) were brought from the Agricultural Institute, Naini, Allahabad (India).

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[1] G.H. Collings, Commercial Fertilizers, p. 393-94 (1954); McGraw Hill Book Company.

[2] E.J. Russell, Soil Conditions and Plant Growth, p. 457, 1950 Edition, Longmans.

[3] G.W., Robinson, W. McLeans, and R. Williams, The Determinations of Organic Carbon in Soils: *J. Agri. Sci.*, **29**, 315 (1929).

[4] D.J. Hissink, Method for Estimating Adsorbed Bases in Soils and the Importance of these Bases in Soils: *Soil Sci.*, **15**, 269-76 (1923).

[5] B. Dyer, On the Analytical Determination of Probably Available 'Mineral' Plant Food in Soils: *Trans. Chem. Soc.*, **65**, 115-167 (1894).

Two handred grams of well-powdered alkali soil, after being passed through a 80 mesh sieve, were taken in shallow enamelled dishes. To these soils 1.0% carbon in one set of Phulpur soil and Soraon soil and 0.5% carbon only in Phulpur soil in another set were added in the form of cow-pea (*Vigna-sinensis*). To these sets 0.25% P_2O_5 in the form of the five Algerian rock phosphates, named, respectively A, B, C, D_1 and D_2 were added. The contents of the dishes were taken in a pestle and mortar and mixed thoroughly to make a homogeneous mixture. About 20% of distilled water was added to each dish. These dishes were exposed to the light of 500 Watt electric bulb hung at a distance of nearly two feet. The contents of the dishes were stirred on alternate days to facilitate aeration and about 20% of water was added on alternate days. Composite samples were taken and analysed for total carbon, exchangeable calcium, available phosphate and pH, respectively.

RESULTS

TABLE I

ANALYSIS OF ALGERIAN ROCK PHOSPHATES FOR TOTAL P_2O_5 AND AVAILABLE P_2O_5

Algerian Rock Phosphate	Total P_2O_5%	Available P_2O_5%
A	29.62	7.57
B	26.45	5.62
C	33.37	6.64
D_1	39.58	3.01
D_2	39.78	3.09

TABLEA II

PERCENTAGE CHEMICAL COMPOSITION OF THE SOILS USED

	Phulpur soil	Soraon soil
1. Moisture	1.3	1.7
2. Loss on ignition	3.2	1.9
3. HCl insoluble	84.19	82.10
4. Sesquioxide	7.05	9.85
5. Fe_2O_3	4.03	1.68
6. CaO	1.20	1.12
7. MgO	1.30	1.73
8. P_2O_5 (total)	0.11	0.13
9. Available P_2O_5	0.017	0.016
10. Total carbon	0.23	0.11
11. Total nitrogen	0.04	0.03
12. Exchangeable Ca	4.5 m.e.%	3.8 m.e.%

Experiments with Soraon alkali soil

Average temperature—30°C

TABLE III

200 g soil+1% C as Cow-pea+0.25% P_2O_5 as Algerian Rock Phosphate (A).

Period of exposure in days	Total Carbon %	Carbon oxidized %	Available P_2O_5 %	Ex. Ca. in m.e. %	pH
0	1.11	—	0.036	3.9	10.3
80	0.72	0.40	0.049	5.3	9.4
160	0.63	0.49	0.070	6.2	8.5
270	0.48	0.63	0.076	6.7	7.4

TABLE IV

200 g soil+1% C as Cow-pea+0.25% P_2O_5 as Algerian Rock Phosphate (B)

Period of exposure in days	Total Carbon %	Carbon oxidized %	Available P_2O_5 %	Ex. Ca. in m.e. %	pH
0	1.11	—	0.028	3.8	10.3
80	0.73	0.38	0.039	5.1	9.6
160	0.65	0.47	0.064	6.0	8.6
270	0.49	0.62	0.07	6.6	7.6

TABLE V

200 g soil+1% C as Cow-pea+0.25% P_2O_5 as Algerian Rock Phosphate (C)

Period of exposure in days	Total Carbon %	Carbon oxidized %	Available P_2O_5 %	Ex. Ca. in m.e. %	pH
0	1.11	—	0.003	3.8	10.3
80	0.73	0.39	0.042	5.2	9.4
160	0.64	0.48	0.067	5.8	8.6
270	0.49	0.62	0.072	6.6	7.5

TABLE VI

200 g soil+1% C as Cow-pea+0.25% P_2O_5 as Algerian Rock Phosphate (D_1)

Period of exposure in days	Total Carbon %	Carbon oxidized %	Available P_2O_5 %	Ex. Ca. in m.e. %	pH
0	1.11	—	0.024	3.8	10.3
80	0.74	0.37	0.033	5.0	9.6
160	0.65	0.46	0.059	5.9	8.6
270	0.50	0.62	0.064	6.6	7.6

TABLE VII

200 g soil+1% C as Cow-pea+0.25% P_2O_5 as Algerian Rock Phosphate (D_2)

Period of exposure in days	Total Carbon %	Carbon oxidized %	Available P_2O_5 %	Ex. Ca. in m.e. %	pH
0	1.11	—	0.025	3.8	10.3
80	0.74	0.38	0.038	5.2	9.5
160	0.65	0.46	0.062	5.9	8.7
270	0.50	0.61	0.064	6.5	7.7

Experiments with Phulpur Alkali soil

Average temperature——30°C

Table VIII

200 g soil+1% C as Cow-pea+0.25% P_2O_5 as Algerian Rock Phosphate (A)

Period of exposure in days	Total Carbon %	Carbon oxidized %	Available P_2O_5 %	Ex. Ca. in m.e. %	pH
0	1.23	—	0.038	4.6	9.6
60	0.78	0.45	0.051	6.2	8.8
120	0.68	0.55	0.069	6.9	7.7

Table IX

200 g soil+1% C as Cow-pea+0.25% P_2O_5 as Algerian Rock Phosphate (B)

Period of exposure in days	Total Carbon %	Carbon oxidized %	Available P_2O_5 %	Ex. Ca. in m.e. %	pH
0	1.23	—	0.028	4.6	9.7
60	0.78	0.46	0.040	5.4	9.1
120	0.72	0.51	0.060	6.0	7.9

Table X

200 g soil+1% C as Cow-pea+0.25% P_2O_5 as Algerian Rock Phosphate (C)

Period of exposure in days	Total Carbon %	Carbon oxidized %	Available P_2O_5 %	Ex. Ca. in m.e. %	pH
0	1.23	—	0.034	4.6	9.7
60	0.79	0.44	0.043	5.7	8.6
120	0.71	0.52	0.067	6.5	7.8

Table XI

200 g soil+1% C as Cow-pea+0.25% P_2O_5 as Algerian Rock Phosphate (D_1)

Period of exposure in days	Total Carbon %	Carbon oxidized %	Available P_2O_5 %	Ex. Ca. in m.e. %	pH
0	1.23	—	0.025	4.6	9.6
60	0.87	0.37	0.030	5.6	8.8
120	0.73	0.50	0.060	6.2	7.9

Table XII

200 g soil+1% C as Cow-pea+0.25% P_2O_5 as Algerian Rock Phosphate (D_2)

Period of exposure in days	Total Carbon %	Carbon oxidized %	Available P_2O_5 %	Ex. Ca. in m.e. %	pH
0	1.23	—	0.026	4.6	9.6
60	0.83	0.40	0.038	5.5	8.8
120	0.71	0.52	0.063	6.2	7.7

Table XIII

200 g soil+0.5% C as Cow-pea+0.25% P_2O_5 as Algerian Rock Phosphate (A)

Period of exposure in days	Total Carbon %	Carbon oxidized %	Available P_2O_5 %	Ex. Ca. in m.e. %	pH
0	0.83	—	0.036	4.6	9.6
60	0.56	0.27	0.048	5.3	8.6
120	0.45	0.38	0.070	6.0	7.8

Table XIV

200 g soil+0.5% C as Cow-pea+0.25% P_2O_5 as Algerian Rock Phosphate (B)

Period of exposure in days	Total Carbon %	Carbon oxidized %	Available P_2O_5 %	Ex. Ca. in m.e. %	pH
0	0.83	—	0.028	4.6	9.6
60	0.51	0.32	0.041	5.4	8.8
120	0.45	0.38	0.058	6.0	7.9

Table XV

200 g soil+0.5% C as Cow-pea+0.25% P_2O_5 as Algerian Rock Phosphate (C)

Period of exposure in days	Total Carbon %	Carbon oxidized %	Available P_2O_5 %	Ex. Ca. in m.e. %	pH
0	0.83	—	0.034	4.6	9.6
60	0.53	0.30	0.040	5.5	8.8
120	0.47	0.36	0.060	6.1	7.8

Table XVI

200 g soil+0.5% C as Cow-pea+0.25% P_2O_5 as Algerian Rock Phosphate (D_2)

Period of exposure in days	Total Carbon %	Carbon oxidized %	Available P_2O_5 %	Ex. Ca. in m.e. %	pH
0	0.83	—	0.026	4.6	9.6
60	0.53	0.30	0.039	5.2	8.9
120	0.46	0.37	0.058	5.8	7.6

DISCUSSION

A close study of the foregoing results clearly shows that carbonaceous material in the form of cow-pea is oxidised more quickly when added to the alkali soil in conjunction with phosphate, such as Algerian Rock Phosphates. In some blank experiments without the addition of cow-pea, it was observed that no change in pH, available phosphate or exchangeable calcium took place even after the lapse of one year.

The oxidation of organic matter like cowpear greatly enhances the availability of insoluble Algerian Rock Phosphates when these phosphates are added in combination with organic materials to the alkali soil. This is due to the reason that tricalcium phosphate, which is present in these Algerian rock phosphates, is converted into more soluble form of phosphates, such as dicalcium phosphate and monocalcium phosphates by the action of carbonic acid produced during the decomposition, ammonification and nitrification of added organic matter. This is clear from the following equations :-

(1) $Ca_3(PO_4)_2+CO_2+H_2O \rightarrow CaHPO_4+CaCO_3$.

(2) $Ca_3(PO_4)_2+2CO_2+2H_2O \rightarrow Ca(HPO_4)_2+2\,CaCO_3$.

The following observations of Dhar6) are of great interest in this connection :-

"The third dissociation constant of phosphoric acid is smaller than the first and second dissociations constants of carbonic acid and hence, carbonic acid converted tricalcium phosphate into dicalcium phosphate, which is more soluble than tricalcium phosphate. Thus, the availability of phosphate is increased in the soil richer in carbonic acid obtained from the oxidation of organic materials. Similarly, nitrous and nitric acids formed in the soil from ammonification and nitrification of proteins make phosphates more readily available. Thus, phosphates are more useful in the presence of organic matter like farmyard manure, straw etc. undergoing slow oxidation".

The above experimental results are also supported by a number of workers. Jensen7) found that the addition of organic matter to a soil increases the solubility of both lime and phosphoric acid from 30-100%. Hester and Shelton8) have also shown that the application of suitable organic matter to the sandy truck soils of the coastal plains of U.S.A. may reduce the fixation of phosphates to an available form so that optimum yields of crop may be produced with much less superphosphate than is commonly used. Copeland and Markle9) and Gerretsen10) have also made similar observations.

The results recorded by us also clearly indicate that the exchangeable calcium of the system is greatly increased when Algerian rock phosphates are added in conjunction with cow-pea. This increase in exchangeable calcium may be ascribed to the dissolving action of carbonic acid and other weak acids, liberated by the oxidation of organic matter, on $CaCO_3$ of the alkali soil or $CaCO_3$ or CaO present in rock phosphates. In this way soluble Ca -ions replace the Na -ions from the exchange complex of the soil, thus, increasing the exchangeable calcium of the system. The carbon dioxide produced may also react with $Ca_3(PO_4)_2$ present in rock phosphate or calcium phosphate present in alkali soil, forming more soluble dicalcium phosphate which introduces more Ca -ions in the system.

In all the cases, the introduction of insoluble phosphates with organic matter greatly helps in lowering the pH of the alkali soil. This is due to the fact that the dicalcium phosphate, formed by the decomposition of organic matter on tricalcium phosphates, When comes in contact with water, partially behaves as a dilute solution of monocalcium phosphate and can reclaim alkali soils slowly.

SUMMARY

The beneficial effect of various sparingly soluble phosphates such as Algerian rock phosphates, when mixed with organic matter

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6) N.R. Dhar, The Value of Calcium Phosphate in Atmospheric Nitrogen Fixation in Soils: *Kung. Lantbruks Annaler*, **21**, 49-78 (1954).

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8) J.B. Hester, and F.A. Shelton, Soil Organic Matter Investigations upon Coastal Plain Soils: *Virginia Truck Expt. Sta. Bull.*, **94**, 1397-1428 (1937).

9) O.L. Copeland, and F.G. Merkle, The Influence of Certain Soil Treatments upon the Fixation and Availability of Applied Phosphates: *Soil Sci. Soc. Amer. Proc.*, **6**, 321-327 (1942).

10) F.C. Gerretsen, The Influence of Micro-organisms on Phosphate Intake by Plants: *Plant and Soil*. **1**, 51-81 (1948).

like cow-pea and added to the alkali soils, is clear from the fall in pH of these alkali soils. Not only the pH is lowered but also the exchangeable calcium, and, the availability of phosphates increases markedly. Hence, these alkali soils ultimately become fertile and cultivable.

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Enzymatic Resolution of Racemic Amino Acids

Part V. Preparation of L-and D-Isomers of Tryptophan, Leucine and Alanine by Enzymatic Resolution of the Acetylderivatives of DL-Amino Acids with Mold Acylase

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The mold acylase of *Aspergillus* and *Penicillium* which hydrolyzes, asymmetrically, only the L-isomer of N-acylated DL-amino acids has been purified previously by the present authors. In this paper the application of asymmetric hydrolysis with the mold acylase to the resolution of N-acylated DL-amino acids, namely, acetylderivatives of DL-tryptophan, DL-leucine and DL-alanine is described. By this enzymatic procedure, the above amino acids were resolved in relatively good yields. It has been noted that the use of the mold acylase is suitable for general resolution of amino acid enantiomorphs of high optical purity.

The purification method of the mold acylase of *Aspergillus* and *Penicillium* which acts asymmetrically on the L-isomer of N-acylated DL-amino acids, has been improved by the present authors[1,2]. It has been noted that the enzyme is relatively easily prepared from the extract of inexpensive molded bran by fractionation with ammonium sulfate and acetone, precipitation with rivanol and elution from the rivanol-enzyme complex. The optical specificity of this enzyme to N-acylated DL-amino acids was previously[1,3] employed for the resolution of several racemic amino acids, namely, DL-valine, DL-glutamic acid, DL-phenylalanine and DL-methione. The present investigation is an extension of this procedure, which is based on the use of the optical specificity of mold acylase, for the resolution of the N-acetylderivatives of DL-tryptophan, DL-leucine and DL-alanine.

EXPERIMENTAL

Enzymatic Assay The purified enzyme solution used for the resolution of acetyl-DL-amino acids was prepared, as described previously[2], by fractionation with ammonium sulfate and acetone, precipitation with rivanol and elution from the rivanol-enzyme complex with phosphate buffer; the final enzyme precipitate obtained by saturation of ammonium sulfate from the above eluate was dissolved in water and dialyzed overnight.

Initial rates of hydrolysis of the N-acetylderivatives* of DL-tryptophan, DL-leucine and DL-alanine with the

TABLE I
INITIAL HYDROLYTIC RATES OF ACETYL-DL-AMINO ACIDS

Substrates	Rate of Hydrolysis		
	Exp. I		Exp. II
Enzyme	0.5 ml.	1 ml.	0.5 ml.
Acetyl-DL-phenylalanine	1.55mM	1.95mM	1.25mM
Acetyl-DL-tryptophan		1.98mM	
Acetyl-DL-leucine		1.00mM	
Acetyl-DL-alanine			1.15mM

The reaction mixture was incubated at 38°C for one hour at pH 7.8. This reaction mixture was composed of 0.5ml of 0.5M acetyl-DL-amino acid, 1ml of 0.1M phosphate buffer of pH 7.8 and 0.5ml (equivalent to 1g of original molded wheat bran, 0.2~0.5mg of solid matter) or 1ml of the purified enzyme solution. The hydrolytic rate was measured by the optical density of ninhydrin reaction of the liberated amino acid expressed in terms of mM of liberated amino acid.

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1) K. Michi and H. Nonaka, This Bulletin, **19**, 153 (1955).

2) K. Michi and H. Tsuda, This Bulletin, **21**, 18 (1957).

3) K. Michi and H. Nonaka, *J. Agr. Chem. Soc. Japan*, **28**, 346 (1954).

*Purchased from General Biochemicals, Institute, U.S.A.

purified mold acylase were determined, and the results are shown in Table I. The pH optima for the hydrolysis of acetyl-DL-tryptophan and acetyl-DL-leucine were found to be approximately pH 7.8 for these acetyl-DL-amino acids. The pH optimum for acetyl-DL-alanine had previously been given as about pH 7.8.

Resolution of Acetyl-DL-tryptophan Ten g (0.041 mol) of acetyl-DL-tryptophan was dissolved in 500 ml of water and neutralized by the addition of 2 g of alcium carbonate to about pH 7. To this aqueous olution, 6 ml (40 mg of solid matter, equivalent to 70 g of molded bran) of purified enzyme was added to insure complete hydrolysis of the L-form of the acetylderivative and the mixture was incubated at 38°C under toluene for two days.

The digest was then treated with aqueous solution of the calculated amounts of oxalic acid to remove calcium. The filtrate through charcoal was evaporated in vacuo to a very small volume, and the residue taken up with alcohol was allowed to stand for 12 hours in a refrigerator. The precipitate, which is almost pure L-tryptophan, was filtered by suction and washed with absolute alcohol. The filtrate was evaporated to dryness and the residue was again taken up with 85% alcohol. When the mixture was allowed to stand in a refrigerator overnight, another crop of L-tryptophan was obtained.

The combined precipitates of L-tryptophan weighed 4 g (87% of theo.). The purified amino acid recrystallized from water showed $[\alpha]^{20}_{\mathrm{D}}-32.3^\circ$ (c 0.5, water). *Analysis*, Found: N, 13.80. Calcd. for $C_{11}H_{12}O_2N_2$: N, 13.72.

Isolation of D-Tryptophan The filtrate of L-tryptophan was evaporated to dryness and the residue was washed with water. The water insoluble-precipitate of acetyl-D-tryptophan was taken up with 100 ml of 2 N sulfuric acid and the solution was refluxed for 2 hours. The solution was diluted with equal volume of water and an aqueous baryta solution was added to remove sulfuric acid. The filtrate through charcoal was evaporated to dryness and the residue taken up with absolute alcohol was allowed to stand in a refrigerator overnight. The precipitate of D-tryptophan of 3 g (65.2% of theo.) was recrystallized from water. The purified D-tryptophan showed $[\alpha]^{20}_{\mathrm{D}}+32.1^\circ$ (c 0.5, water).
Analysis, Found: N, 13.85. Calcd. for $C_{11}H_{12}O_2N_2$: N, 13.72.

Resolution of Acetyl-DL-leucine Ten g (0.058 mol) of acetyl-DL-leucine were dissolved in 500 ml of water and after addition of 2.8 g of calcium carbonate the mixture was incubated with an appropriate amount of purified enzyme (150 mg of solid matter, equivalent to 250 g of molded bran) at 38°C., for two days.

The digest was treated with aqueous solution of oxalic acid to remove calcium. The filtrate was evaporated to crystallize L-leucine, warmed with 3 volumes of absolute alcohol and the mixture was allowed to stand in a refrigerator. L-Leucine thus obtained, amounted to 3.3 g (87% of theo.) and was recrystallized from water. L-Leucine showed $[\alpha]^{20}_{\mathrm{D}}+15.65^\circ$ (c 4, 6 N HCl). *Analysis*, Found: N, 11.00. Calcd. for $C_6H_{13}O_2N$: N, 10.68.

Isolation of D-Leucine The combined filtrates which had been separated from the L-leucine preparation were evaporated to dryness and taken up with a small amount of water. After standing in a refrigerator the crystallized acetyl-D-leucine was filtered. The precipitate which showed m.p. 185°C. and a negative ninhydrin reaction (5.5 g of wet substance) was refluxed with 100 ml of 2 N sulfuric acid for two hours. The digest was treated with baryta to remove sulfuric acid as in the case of tryptophan. The filtrate was evaporated to dryness and the residue was taken up with absolute alcohol. The precipitate of D-leucine was washed with alcohol. It weighed 2.8 g (74% of theo.) and was recrystallized from water. It showed $[\alpha]^{20}_{\mathrm{D}}-15.35^\circ$ (c 4, 6 N HCl). *Analysis*, Found: N, 10.67. Calcd. for $C_6H_{13}O_2N$: N, 10.68.

Resolution of Acetyl-DL-alanine Ten g (0.077 mol) of acetyl-DL-alanine were dissolved in 500 ml of water and neutralized with 3.5 g of calcium carbonate to pH 7. An appropriate amount of acylase solution (70 mg in 10 ml of water, corresponding to 120 g of molded bran) was added to the solution and the mixture was incubated under toluene at 38°C., for two days.

At the end of the enymatic hydrolysis, the digest was treated with oxalic acid as described in the case of tryptophan. The filtrate was evaporated in vacuo to about 20 ml and, after addition of absolute alcohol to 65% content, the mixture was allowed to stand in a refrigerator for 12 hours. The precipitate of L-alanine which weighed 2.8 g (81% of theo.) was filtered and recrystallized from a water-alcohol mixture. $[\alpha]^{20}_{\mathrm{D}}+14.63^\circ$ (c 6, 1 N HCl). *Analysis*, Found: N, 15.69. Calcd. for $C_3H_7O_2N$: N, 15.72.

Isolation of D-Alanine The filtrate which had been separated from L-alanine preparation was evaporated and the residue was dissolved in absolute alcohol-acetone mixture and allowed to stand in a refrigerator. The precipitate was removed and the solution was evaporated. The residue which was almost pure acetyl-D-alanine was refluxed with 80 ml of 2 N sulfuric acid for 3 hours.

The mixture was treated with baryta solution to remove sulfuric acid as described in the case of tryptophan. After evaporation of the filtrate, the residue was treated with alcohol. The precipitate of D-alanine was filtered and recrystallized from 60% alcohol. The yield of D-alanine was 2.4 g. (71% of theo.) $[\alpha]_D^{20}$ − 14.10° (c 6, 1 N HCl). *Analysis*, Found: N, 15.87. Calcd. for $C_3H_7O_2N$: N, 15.72.

DISCUSSION

During the past several years, Greenstein et al.[4-9] have accomplished the preparation of D- and L-isomers of most of the amino acids on a large scale by using renal acylase for the resolution of acylderivatives of all α-amino acids except for the aromatic amino acids which are best resolved by employing pancreatic carboxypeptidase instead of the renal acylase.

The present authors have so far resolved seven DL-amino acids, namely, DL-valine, DL-glutamic acid, DL-phenylalanine, DL-methionine, DL-tryptophan, DL-leucine and DL-alanine by substituting the mold acylase for the renal acylase and the carboxypeptidase. The present paper is concerned with the enzymatic resolution of acetylderivatives of the latter three racemic amino acids.

The initial hydrolytic rates of N-acetyl-DL-tryptophan, N-acetyl-DL-leucine and N-acetyl-DL-alanine were compared with that of acetyl-DL-phenylalanine which was best resolved previously, by the use of mold acylase. Acetyl-DL-tryptophan is susceptible for the mold acylase in the same degree of hydrolytic rate of acetyl-DL-phenylalanine as shown in Table I. Acetyl-DL-alanine is also a susceptible substrate for the mold acylase. The initial rate of hydrolysis which had been previously[3] measured had in a lower value. The repeated

TABLE II

ENZYMATIC PREPARATION OF L-AND D-AMINO ACIDS BY ASYMMETRIC HYDROLYSIS OF N-ACYLDERIVATIVES OF DL-AMINO ACIDS WITH MOLD ACYLASE

DL-Amino Acid	Derivative employed	Amino Acid resolved	Yield %	°C	$[\alpha]_D$	Data* in literature
DL-Valine	Chloroacetyl	L-Valine	89	24	+27.7 (3.4% in 6 N HCl)	+27.4[5]
		D-Valine	72	24	−27.6 (〃)	−27.1[5]
DL-Glutamic Acid	Acetyl	L-Glutamic acid	95	28	+31.3 (2.08% in 6 N HCl)	+32.0[5]
		D-Glutamic acid	64	28	−31.1 (〃)	−31.9[5]
DL-Phenylalanine	Acetyl	L-Phenylalanine	83	20	−34.8 (1.6% in water)	−34.8[6]
		D-Phenylalanine	70	20	+34.6 (〃)	+34.8[6]
DL-Methionine	Acetyl	L-Methionine	86	14	+23.42 (2% in 6 N HCl)	+23.4[10]
		D-Methionine	61	14	−23.3 (〃)	−23.4[10]
DL-Tryptophan	Acetyl	L-Tryptophan	87	20	−32.3 (0.5% in water)	−32.2[6]
		D-Tryptophan	65	20	+32.1 (〃)	+32.5[6]
DL-Leucine	Acetyl	L-Leucine	87	20	+15.65 (4% in 6 N HCl)	+15.9[5]
		D-Leucine	74	20	−15.35 (〃)	−15.6[5]
DL-Alanine	Acetyl	L-Alanine	81	20	+14.63 (6% in 1 N HCl)	+14.4[5]
		D-Alanine	71	20	−14.10 (〃)	−14.4[5]

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4) J.P. Greenstein et al., *J. Biol. Chem.*, **178**, 503 (1949).

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10) M. Brenner, *Helv. Chim. Acta.*, **32**, 333 (1949).

*Data in the literature for all amino acids except methionine are those reported by Greenstein et al.

measurement of the hydrolytic rate of acetyl-DL-alanine shows a rather high value. When compared with other amino acids, acetyl-DL-leucine is relatively resistant for hydrolysis by the mold acylase. Therefore, the 2.5-fold amount of the enzyme for acetyl-DL-tryptophan must be employed for the resolution of acetyl-DL-leucine in order to insure complete hydrolysis. The amino acids which were so far resolved by the enzymatic procedure with the mold acylase by the present authors are listed in Table II.

The enzymatic resolution procedure with the mold acylase showed good yields of the L- and D-isomers of the amino acids which varied from 60 to 95% of the theoretical. Each amino acid is checked for its optical purity by measuring the optical rotation. As shown inTable II, the specific rotation of each amino acid was compared with those in the literature and these were found to be in good agreement with those given by Greenstein et al.5,6,10)

Since it became available to easily prepare the purified enzyme from inexpensive molded wheat bran as described previously, it seems that the use of mold acylase for the resolution of DL-amino acids can be extended to the resolution of all other racemic amino acids. This series of study is under way.

Acknowledgment The authors are grateful to Dr. S. Abe and Dr. H. Iizuka of the Microbiology Institute of University of Tokyo for kindly supplying *Penicillium* and *Aspergillus* and Miss Isobe for performing the microanalyses. The research was aided by a Grant from the Ajinomoto Company.

SUMMARY

(1) The enzymatic procedure of the asymmetric hydrolysis of N-acylated DL-amino acids with the mold acylase was applied for the resolution of DL-tryptophan, DL-leucine and DL-alanine.

(2) Acetylderivatives of DL-tryptophan, DL-leucine and DL-alanine were incubated at 38°C. for 2 days, at approximately 0.1 M concentration in water with an appropriate amount of enzyme solution which was prepared from the molded bran.

(3) By this procedure, L-isomers of each amino acid were liberated and the corresponding D-isomers were obtained by acid hydrolysis of the residual acetyl-D-amino acids. Thus, the optical enantiomorphs of tryptophan, leucine and alanine were prepared with a high degree of optical purity.

(4) Acetyl-DL-tryptophan was best resolved by the asymmetric hydrolysis with mold acylase and acetyl-DL-alanine was also a susceptible substrate for mold acylase. Acetyl-DL-leucine was relatively resistant to the enzyme, as compared with the other acylated amino acids so far resolved. An amount of enzyme two and a half times as large as that for acetyl-DL-tryptophan was required for acetyl-DL-leucine.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 4, p. 239~242, 1957]

Studies on the Essential Oils of Tobacco Leaves

Part XV. The Neutral Fraction (2)

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Benzyl alcohol, β-phenylethyl alcohol and methyl alcohol were isolated and identified as free alcohols from the Neutral fraction of the essential oil of the aged Japanese Flue Cured Tobacco Leaf. On the other hand, benzyl acetate and β-phenylethyl acetate were both isolated each in the ester form and identified, respectively. Besides these esters, the presence of methyl laurate, methyl myristate and methyl palmitate were also confirmed in their ester form. All these compounds, except methyl alcohol, were newly isolated and identified compounds from tobacco plant and they seemed to be responsible for the aroma and taste of excellent Virginia tobacco leaf.

In the previous paper[1], the isolation and identification of α-pyrrylmethylketone, ethyl alcohol and furfuryl alcohol together with the estimation of C_4-alcohol and an unsaturated hydrocarbon, resembling myrcene, in the Neutral fraction of the essential oil of the aged Japanese Flue Cured Tobacco Leaf (JFCTL) were reported. Further investigation of this fraction has been conducted. The low boiling point-fraction, illustrated as the α-fraction in the previous Table III, was derived directly into 3,5-dinitrobenzoate (3,5-DNB) and separated by a liquid-chromatography.

A small amount of methyl alcohol was isolated and identified besides the large amount of ethyl alcohol, reported previously. After the removal of the low boiling point-fraction, hydrocarbons were separated by liquid chromatography and thereafter, an oxygenated compound-fraction was eluted out with ether.

Subsequently, the oxygenated compounds were fractionally distilled. A fraction was reacted with 3-nitrophthalic anhydride and free alcohol derivatives were isolated. The half-ester obtained was saponified and the free alcohols separated were derived successively into 3,5-DNB in order to separate them by liquid-chromatography. A crystal of the free alcohol derivative obtained in this manner was separated into two parts by a fractional recrystallization and the one was identified as benzyl alcohol while the other as β-phenylethyl alcohol.

The residual neutral-fraction, after the free alcohols had been separated, was successively separated by a liquid-chromatography. From an early stage of elution, a fraction was obtained which had been estimated to be some acetic acid ester of aromatic alcohol by an infrared spectrum. Consequently, the ester was saponified. From the alcoholic-fraction, β-phenylethyl alcohol and benzyl alcohol were identified by the method as described in the case of free alcohols. The acid-fraction from the ester was derived into hydroxamic acid and developed on paper-chromatography[2]. Neither spot could be observed except one which indicated an R_F value agreeing with that of acetic acid. Thus, the presence of β-phenylethyl acetate and benzyl acetate which had been estimated by the former infrared spectra was confirmed.

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[1] I. Ōnishi, H. Tomita and T. Fukuzumi; This Bulletin, **20**, 61 (1956).

[2] Y. Inoue and M. Noda, *J. Agr. Chem. Soc Japan*, **24**, 291 (1950).

On the other hand, a pale lemon-yellow color band which makes the chromatographic column semi-transparent was separated, prior to the separation of the above mentioned oxygenated compounds. Infrared spectrum of this eluate showed that this band might be some methylester of higher fatty acid. An aliquot of this band reacted directly with 3,5-dinitrobenzoyl chloride[3] and methyl alcohol alone was identified. Another aliquot was saponified and the acids obtained were derived into 2,4-dinitrophenylhydrazide. Thus, palmitic acid, myristic acid and lauric acid were identified by the reverse phase chromatography[4]. Consequently, the presence of methyl palmitate, methyl myristate and methyl laurate was confirmed.

EXPERIMENTAL

I) Identification of methyl alcohol. The low boiling point-fraction was derived into 3,5-DNB and the crystal obtained was separated by liquid-chromatography[1]. A faint band, following a strong one which had been identified as ethyl alcohol, was separated. In removing the eluting solvent from the faint band a flaky white crystal was obtained. After recrystallization from *n*-hexane, containing a small amount of ethyl alcohol, the mixed melting point of this crystal with the authentic 3,5-DNB of methyl alcohol showed no depression and both infrared spectra agreed well with each other.

II) Identification of benzyl alcohol and β-phenylethyl alcohol. The infrared spectrum of the high boiling point-fraction (b.p. 88–120°/2.3 mm, 3.8050 g/227 kg of tobacco leaf) of the oxygenated compounds showed very strong absorption of free alcohol. Consequently, 3-nitrophthalic anhydride was added and reacted on an oil bath for forty-five minutes at 100–115°. The half-ester resulted was extracted with five per cent sodium carbonate. The extract thus obtained was saponified by an aqueous solution of caustic alkali and extracted with ether. In removing ether, the residue was derived into 3,5-DNB and separated by liquid-chromatography. Three bands were separated. A large amount of white crystal was obtained from the middle band. After recrystallization from a mixture of ethanol and *n*-hexane, a white needle crystal, m.p. 113.0–114.2°, and another light-yellow flaky crystal, m.p. 108.0–108.4°, were obtained. The mixed melting point of the former white needle crystal (0.73 mg/1 kg of leaf, as free alcohol) with the authentic 3, 5-DNB of benzyl alcohol showed no depression and both infrared spectra agreed completely with each other. *Anal.* Found: C, 55.87; H, 3.15; N, 9.52. Calcd. for $C_{14}H_{10}N_2O_6$: C, 55.63; H, 3.34; N, 9.27.

On the other hand, the mixed melting poing of the latter light-yellow flaky crystal (0.37 mg/1 kg of leaf, as free alcohol) with the authentic 3,5-DNB of β-phenylethyl alcohol showed no depression and both infrared spectra agreed completely with each other. *Anal.* Found: C, 57.50; H, 3.87; N, 8.80. Calcd. for $C_{15}H_{12}N_2O_6$: C, 56.96; H, 3.83; N, 8.86.

Besides these results, both the top and the bottom bands showed a strong yellow spot on each chromatostrip when sprayed with α-naphthylamine, indicating that this spot consisted of 3,5-DNB of other kinds of alcohol. However, they gave only a small amount of oily extracts, so that they could not be identified.

III) Identification of β-phenylethyl acetate and benzyl acetate. Following removal of the free alcohols as described above (II), the residual fraction was successively separated by liquid-chromatography. From an early stage of elution, an oily substance was obtained. A chromatogram of this substance on a chromatostrip, when developed with *n*-hexane containing 15% ethyl acetate and sprayed with a mixed acid[1], showed a light-yellow spot of R_F value 0.55–0.60. The infrared spectrum of this substance showed a specific absorption of ester (1740 and 1250 cm^{-1}) and mono-substituted aromatic constitution in its alcoholic moiety (700 and 750 cm^{-1}). Subsequently, the substance was saponified by alcoholic potash and the neutral fraction obtained was derived into 3,5-DNB and purified by liquid-chromatography. A white needle crystal was obtained from this eluate. Then, it was divided into two crystals by a successive fractional recrystallization. The one melted at 113.8–114.2° and the other 107.8–108.2°. Both crystals were identified as 3, 5-DNB of benzyl alcohol and β-phenylethyl alcohol by their mixed melting points and infrared spectra with their authentic compounds, respectively.

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[3] W.B. Renfrow and A. Chaney; *J. Am. Chem. Soc.*, **68**, 150 (1946).

[4] Y. Inoue and M. Noda; This Bulletin, **19**, 214 (1955).

After removal of the alcoholic fraction the saponified product was acidified with sulfuric acid and extracted with ether succeeded by a saturation with sodium chloride. The solvent was carefully removed and the distillation residue obtained had a strong acidic odor of low molecular fatty acid. Then, it was derived into hydroxamic acid and investigated on a paper chromatography, employing a mixture of *n*-butanol (saturated with water) and benene (1 : 1 by volume), as a developing solvent. One spot of acetic acid alone was observed on the paper chromatogram, so that esters mentioned above were identified as β-phenylethyl acetate (0.98 mg/1 kg of leaf) and benzyl acetate (0.63 mg/1 kg of leaf), respectively.

IV) Identification of methyl ester of higher fatty acids. On removal of hydrocarbons from the neutral fraction by liquid-chromatography on the silicic acid column, employing *n*-hexane as an eluting solvent, the eluting solvent was changed to a mixture of *n*-hexane and ether (100 : 0.5–1.0 by volume) and a semi-transparent band was separated. In removing the solvent from an eluate of this band a white waxy substance was obtained on cooling (6.4 mg/1 kg of leaf). This substance dissolved in both ethanol and acetone at room temperature and solidified on cooling. An infrared spectrum of this substance agreed with that of a methyl-ester of higher fatty acid. An aliquot of it was derived directly into 3,5-DNB[3] and it was purified by successive liquid-chromatography. From an eluate of the major band a flaky crystal, m.p. 108.2–108.5°, was obtained. According to its infrared spectrum and mixed melting point, it was identified as 3,5-DNB of methyl alcohol. On the other hand, an R_F value of an eluate of the minor band, which was eluted before the major band, agreed well with that of the same kind of derivative of ethyl alcohol on a chromatostrip. However, because of its small quantity, no crystal was obtained.

Another aliquot of the above mentioned waxy substance was saponified by an alcoholic potash and then extracted with ether after the reaction medium acidified strongly with sulfuric acid. Removal of the ether from the extract left a white waxy substance. The infared spectrum of this substance agreed well with that of a higher fatty acid. Consequently, it was derived into 2, 4-dinitrophenylhydrazide and developed on a reverse phase chromatography. From the R_F values of the separated spots they were identified as palmitic acid, myristic acid and lauric acid, respectively. A spot of stearic acid was observed faintly but its presence was not identified because of its very small quantity. According to these results, the constituents of the above mentioned waxy substance were identified as methyl palmitate, methyl myristate and methyl laurate, respectively. The proportions of these esters were estimated as 2 : 3 : 2, by the area of spots on the paper chromatogram.

DISCUSSION

Formerly, the investigation on esters has usually been conducted in such a manner that they were saponified simultaneously and some possible pair of combinations have been suggested by both moieties of separated acids and alcohols. However, concerning the study of tobacco aroma, it seemed preferable to make the combination of these moieties as clear as possible.

In this study, benzyl acetate and β-phenylethyl acetate were identified by the experimental results, in which the acid moiety of these esters consisted of acetic acid alone, whereas, in the case of esters of higher fatty acids, it could be clarified that the alcoholic moiety was consisted mainly by methyl alcohol and the amount of other alcoholic constituents was found negligible. Consequently, the combinations of both moieties of esters were clearly identified and these results might contribute greatly for the further investigations of tobacco flavours.

Benzyl alcohol and β-phenylethyl alcohol and their acetates, isolated and identified in this study, are widely found in many other plants and contribute much to the excellent aroma of the essential oils with their light floral scent (rose character) and fruity odor suggesting a jasmine oil (rose honey character).

Although the amounts of these aromatic constituents are not so large, they seemed much responsible for the smoking aroma in comparison with those of such individual element practically used of tobacco flavouring materials. Furthermore, as the abovementioned amounts were found from those of the final crystals, much loss in the process of isolation and purification is not included in these amounts. Consequently, it is assured that the tobacco leaf contains larger amounts of these aromatic constituents practically than the above mentioned ones.

On the other hand, these compounds mentioned above are frequently used in many artificial flavours. However, in regard to the tobacco flavours, differing from many other ones, particular attention must be drawn upon the criteria that they have been required to possess not only good aroma but also good taste in smoking. In this respect, it seemed very interesting to note that the above-mentioned constituents contribute either to the aroma or the taste in smoking.

In general, it is considered that, when the higher fatty acids and their esters are applied as casing materials in tobacco manufacturing, the strength of smoke turns much milder and smoking quality may be improved much more. In this respect, the isolation and identification of these esters of higher fatty acids seemed to be very interesting. Especially, the isolation and identification of myristic acid ester in tobacco leaf are supposed to be worthy of note concerning the aroma and taste of tobacco smoke.

On the other hand, the changes of the quantity of methyl alcohol during fermentation and aging, has once been considered as one of the most important keys suggesting the degree of aging of tobacco leaves[5~8]. However, the amount of methyl alcohol in the essential oil of aged Japanese flue cured tobacco leaf was proved to be so small that it seemed very difficult to trace the difference of its amounts between the pre-redrying and after-aging periods.

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Sulfur Metabolism in Higher Plants

1. The Conversion of Sulfate into Organic Sulfur in Excised Leaves

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The contents of organic sulfur in various seedlings did not increase but rather decreased during the cultivation-period within 8 to 12 days. In mung bean seedling, the synthesis of organic sulfur from sulfate took place only in the leaves. Organic sulfur in the roots and stems may be transported from the cotyledons. Even in the excised leaves, the rapid conversion of sulfate into organic form took place both in air and reduced pressure. The organic sulfur compound synthesized during the incubation for the short period was cystine or cysteine and methionine was detected only after a long time. It may be possible that this system is employed in studies on the pathway of the conversion of sulfate into organic form.

Although sulfur is an essential constituent of higher plants, only limited information is available in the literature concerning biochemical studies on the sulfur metabolism in higher plants. Thomas et al. found that, in wheat, barley and corn, the radioactive sulfur, which was supplied either as sulfur dioxide through the leaves or as sodium sulfate through the roots, was converted very rapidly into the organic form which appeared to be cystine or methionine[1]. The conversion of radioactive sulfate into the organic form was also observed in wheat seedling by Kylin[2]. Further, Thomas et al. found that, in alfalfa, sulfate-sulfur gradually changed into sulfur of the organic compounds contained in leaves[3].

In microorganisms, although no experiments on the enzymatic level have been performed and only indirect methods with the use of intact cells have been employed, many researchers have reported the data and speculations concerning the mechanism of the conversion of sulfate-sulfur into organic sulfur. For example, Cowie et al. from the studies by the tracer techniques suggested that, in *Escherichia coli*, sulfite and thiosulfate were the intermediates in the pathway of the biosynthesis of sulfur in the organic form from sulfate, while taurine and cysteic acid were not the intermediates[4].

On the contrary, in spite of rapidly converting sulfate into the organic form in plant-tissues as described above, no information concerning this mechanism is yet available in higher plants; this may be due to the fact that intact plants are too complicated by the number of various tissues and organs which are to be employed for the biochemical studies elucidating this mechanism. Thus, in higher plants, a simpler system capable of converting sulfate into the organic form must be established.

In this paper, the simpler system of living cells, which is available in the studies concerning the mechanism of the conversion of sulfate into organic compounds, and the several results obtained from the studies with this system are described.

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MATERIALS AND METHODS

Cultivation of Plants Some species of the seeds were grown in a basin, its bottom covered with filter paper with occasional additions of the nutrient solution. The temperature was kept at 25–30°C throughout the whole period of cultivation and an artificial light of 4000–6000 Lux., was supplied for 10 hr. daily. After 4 or 5 days, the seedlings were washed with distilled water, and they were then grown again according to the above method. In case, where only the leaves were employed for studies, the primary leaves were excised after cultivation for 10–13 days; at this time the seedlings have no leaves expect the primary leaves in mung bean (*Phaseolus radiatus* var. *typica Prain*).

Determination of Sulfur In the experiment determining the amount of sulfate-sulfur, the following method was applied. About 1 g of the sample, which previously had been weighed, was homogenated with HCl (1 : 2.5) and then the protein was removed by the addition of 4 volumes of alcohol and centrifugation. The supernatant was dried up on the water bath, heated at 110°C for 1 hr., and extracted with 1 N HCl. The extract in which the insoluble materials had been removed by filtration was filled up to 10 ml and 1 ml of this solution was pipetted into a 15 ml centrifuge-tube. The sample in the centrifuge-tube was neutralized, to which 0.5 ml of 0.5% uranyl acetate were added. After mixing with 0.01 M $BaCl_2$, the mixture was allowed to stand for several hours. One ml of 10% sodium acetate and 2 ml of 0.2% $K_2Cr_2O_7$ were added at a low temperature and the resulting precipitate was dissolved by the addition of 2 ml of 2 N HCl. The content of perchromate ion in the solution was determined by titration with a standardized solution of Mohr's salt.

In determining the amount of total sulfur, about 1 g of the sample was heated with Na_2O_2 and Na_2CO_3 and was dissolved in water[5]. An aliquot of the solution was put into the centrifuge-tube and then the amount of sulfate in the tube was determined by the above method.

Incubation of Excised Leaves The excised leaves previously washed with distilled water was submerged into the carrier-free solution of S^{35}-labeled sulfate (2–5 μc/ml). By penetration in vacuum, isotope was infiltrated into the leaves. After washing the leaves with water in order to remove the isotope adhering on their surface, the leaves were submerged into water and incubated in a desicator at 30°C.

Measurement of Radioactivity The radioactivity of sulfate-sulfur was measured by the following method. One gram of the sample was extracted with 5% trichloroacetic acid and the soluble fraction was obtained by centrifugation. To an aliquot of the supernatant which was neutralized by alkali and then mixed with Na_2SO_4 as the carrier, 5 ml of 10% $BaCl_2$ were added and the resulting precipitate was collected by the centrifugation. After the precipitate was washed with water and alcohol, it was translocated into a counting plate with alcohol and dried, the radioactivity of which was counted by the usual method.

The radioactivity of total sulfur was measured by the above method, after total sulfur in the sample had been converted to sulfate-sulfur by heating with Na_2O_2 and Na_2CO_3.

In the measurement of the radioactivities on spots of the paper chromatography, the dried paper was fractioned to circular fragments of 2.2 cm in diameter, which were placed in the counting plates. In this case, the decrease of radioactivity by the absorption of β-ray was neglected.

Paper Chromatography The excised leaves, which had been incubated with the method described above, was homogenated with 10% trichloroacetic acid and the soluble fraction, which was separated by the centrifugation, was neutralized with alkali to be spotted on the paper and developed with the mixture of butanol, acetic acid and water (4 : 2 : 1). The substances of R_F value higher than 0.10 were extracted with 0.1 N HCl. The extract was concentrated under reduced pressure and spotted on the paper to be followed by developing with the various solvents.

RESULTS

The Change of Amounts of Sulfur in Organic Form in Various Seedlings In the studies on the mechanism of conversion of sulfate into organic form, it is reasonable to employ the plant, which is able to convert sulfate rapidly into the organic form and grow by the simpler cultivating method, as the sample. Thus, the change of contents of sulfur in organic form in the various seedlings was determined. Representative data of a series of these experiments are shown in Fig. 1., where the content of sulfur in each form is represented

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[5] Official Method of Analysis, A.O.A.C., 1950, p. 103.

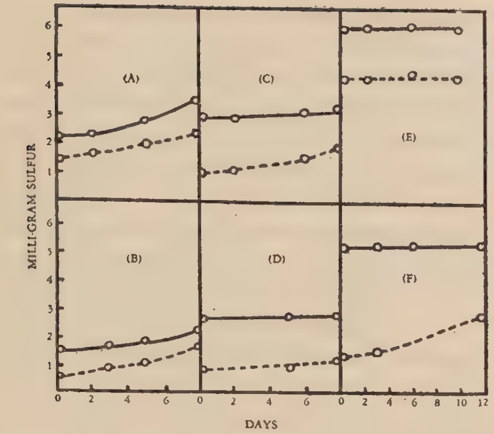

FIG. 1. The Change of the Contents of Sulfur during the Period of Cultivation in Various Seedlins.

Solid lines; contents of total sulfur
Broken lines; contents of sulfate-sulfur
A; in mung bean B; in wheat C; in azuki bean
D; in tomato E; in cabbage F; in welsh onion

as the amount of sulfur in the seedlings obtained from 1 g of seeds. It can be thought that the difference between the amounts of total sulfur and salfate-sulfur corresponds to the amount of sulfur in the organic form. In most of the seedlings, the contents of sulfur in the organic form did not increase but rather decreased within the period of cultivation, while in mung bean the content slightly increased. Furthermore, since in mung bean it is possible that the simpler cultivating method is applied and a large number of seedlings are easily obtained, the seedlings, therefore, are the best sample of the various seedlings which were studied in our laboratory. Thus, in all the following experiments, mung bean was employed as the sample.

Content of Sulfur in Each Organ The amounts of sulfate-sulfur and total sulfur in each organ of mung bean seedlings in the age of cultivation for 11 days are summarized in Table I. The content is represented as the amount of sulfur in each organ of seedlings obtained from 1g of seeds. An amount of 0.2 mg of sulfur in the organic form increased for the period of cultivation. In leaves, roots and stems, and cotyledons, the ratio of content of organic sulfur to one of total sulfur was 0.33, 0.23 and 0.22, respectively. Most of the sulfur existing in whole seedlings were present in the roots and stems.

TABLE I
CONTENT OF SULFUR FOUND IN EACH ORGAN

Organ	Total sulfur	Sulfate-sulfur	Organic sulfur
	mg	mg	mg
Seed	2.3	1.6	0.7
Whole	3.8	2.9	0.9
Leaf	0.6	0.4	0.2
Root, Stem	2.2	1.7	0.5
Cotyledon	0.9	0.7	0.2

Fate of Radioactive Sulfate in the Seedling The fate of radioactive sulfate in the seedlings of mung bean was studied and the results are shown in Tables II and III, in which the value represents the radioactivity of sulfate-sulfur in a seedling. In those experiments, radioactive sulfur was supplied as sulfate through the roots and cotyledons for 24 hr. after cultivating for 4 days. It can be considered that the difference between the radioactivities of total sulfur and sulfate-sulfur corresponds to the radioactivity of synthesized organic sulfur. This difference, as seen in Table II., could not be detected in the analyses of the whole plant-body. While, in the measurement of radioactivity in each organ of the seedlings at the age of cultivation for 6 days after infiltrating isotope, radioactive organic sulfur was detected only in the leaves, as shown in Table III. In addition, the ratio of radioactivity of organic sulfur to that of total sulfur in this organ was 0.34, which is equal to the ratio of content of organic sulfur to that of total sulfur as seen in Table I. This indicates that an equilibrium in the interconversion between radioactive sulfate-sulfur and radioactive organic sulfur was established at the time.

The majority of the absorbed isotope was present in roots and stems and only small amounts of it were found in leaves. It may be considered that, owing to this relation, radioactive organic sulfur could not be detected in the experiment with the whole plant as shown Table II.

TABLE II
CHANGE OF THE AMOUNT OF RADIOSULFATE IN THE WHOLE SEEDLING

Days after infiltration	Radioactivity of total sulfur	Radioactivity of sulfate-sulfur
	c.p.m.	c.p.m.
0	7.5×10^3	7.8×10^3
2	7.7×10^3	7.8×10^3
4	7.8×10^3	7.8×10^3
6	7.5×10^3	7.5×10^3

TABLE III
CHANGE OF RADIOSULFATE IN EACH ORGAN

Organ	Radioactivity of total sulfur	Radioactivity of sulfate-sulfur
	c.p.m.	c.p.m.
Leaf	0.38×10^3	0.25×10^3
Root and Stem	1.82×10^3	1.82×10^3
Cotyledon	0.91×10^3	0.89×10^3

The Conversion of Sulfate in Organic Form in Excised Leaves The establishment of the simpler system of living cells capable of synthesizing organic sulfur compounds from sulfate was attempted. The primary leaves having this ability were excised, into which radioactive sulfate was infiltrated, in vacuum. After washing them with water and then incubating in water for 3 hr., their radioactive sulfur content was analyzed. The results, summarized in Table IV, show that the rapid synthesis took place in the various conditions of incubation. The more rapid conversion of sulfate into the organic form in the anaerobic condition may be explained by the assumption that sulfite or sulfide, considered as the possible intermediate, may be hardly oxidized in this condition. Illumination of artificial light of 4000 Lux. did not increase the grade of incorporation of sulfate-sulfur into organic compounds.

TABLE IV
CONVERSION OF SULFATE INTO THE ORGANIC FORM IN EXCISED LEAVES

Condition of incubation*	Radioactivity of total sulfur**	Radioactivity of sulfate-sulfur**	Grade of incorporation***
	c.p.m.	c.p.m.	%
I	2.18×10^4	1.41×10^4	38
II	2.01×10^4	1.18×10^4	41
III	2.07×10^4	1.02×10^4	51
IV	1.90×10^4	0.92×10^4	51

*Under conditions I, II, III and IV, leaves were incubated in the air and in the light, in the air and in the dark, in reduced pressure and at light, and in reduced pressure and at dark, respectively.
**The values represent the radioactivities in 1g of leaves.
***The values represent the percentage values of radioactivities of organic sulfur to those of total sulfur.

Tables V and VI show the evidences that the rapid conversion of radioactive sulfate into the organic form in excised leaves was independent of the contamination of microorganisms. The maximal grade of the conversion of sulfate into organic form was accomplished at the incubation for 3 hr. (Table V) and this relation was strengthend by the experiments with paper chromatography, in which it was shown that the content of radioactive organic sulfur reached the maximum within 1 hr.. In addition to the very rapid conversion, the grade of incorporation did not decrease even in the

TABLE V
RATE OF CONVERSION OF SULFATE INTO THE ORGANIC FORM IN EXCISED LEAVES

Time	Radioactivity of total sulfur	Radioactivity of sulfate-sulfur	Grade incorporation
hr.	c.p.m.	c.p.m.	%
3	5.03×10^4	3.81×10^4	24
6	5.44×10^4	4.23×10^4	22

TABLE VI
EFFECTS OF ANTIBIOTICS TO THE INCORPORATION OF SULFATE INTO ORGANIC FORM IN EXCISED LEAVES

Antibiotics	Radioactivity of total sulfur	Radioactivity of sulfate-sulfur	Grade of incorporation
	c.p.m.	c.p.m.	%
No addition	5.38×10^4	4.93×10^4	9
Streptomycin	7.50×10^4	6.53×10^4	13
No addition	5.03×10^4	3.81×10^4	24
Aureomycin	5.47×10^4	4.20×10^4	23

concentration of streptomycin or aureomycin, sufficient to inhibit growth of microorganisms, as shown in Table VI. These results eliminate the suspicion that the synthesis of sulfur-containing organic compounds may take place as a results of contamination with microorganisms.

The attempt that leads to synthesize the sulfur-containing organic compounds using the homogenate of leaves was carried out in replication, but resulted in fail.

Identification of Synthesized Sulfur-Containing Organic Compound The identification of synthesized sulfur-containing organic compounds was attempted by the technique with paper chromatography. Representative data of a series of these experiments are shown in Fig. 2. In all cases where the various solvents were employed as the developing sovlents, only a radioactive organic compound was detected after incubation for 3 hr., and the R_F of this compound was accordant with one of the exogenously added cystine which was detected by coloring with nitroprusside-reagent[6]. The radioactive substance on the original point may be radioactive sulfate. These results show that the synthesized sulfur-containing organic compounds is perhaps cystine or cysteine. Radioactive methionine was not detected in both the acid-soluble fraction and the hydrolysate of the acid-insoluble fraction.

The change of neosynthesized sulfur-containing organic compounds in the various periods of incubation was studied. Representative data of a series of these experiments are shown in Fig. 3. In all cases, the whole acid-soluble fraction was spotted and the mixture of butanol, acetic acid and water was employed as the developing solvent. Cystine or cysteine was detected even in the incubation for 1 hr. and moreover the ratio of radioactivity of this compound to one of sulfate-sulfur after incubation for 1 hr. was apparently equal to one after incubation for 3 hr.. These results indicate that the rapid incorporation of sulfate into organic sulfur took place in this system. In addition of cystine of cysteine, another radioactive sub-

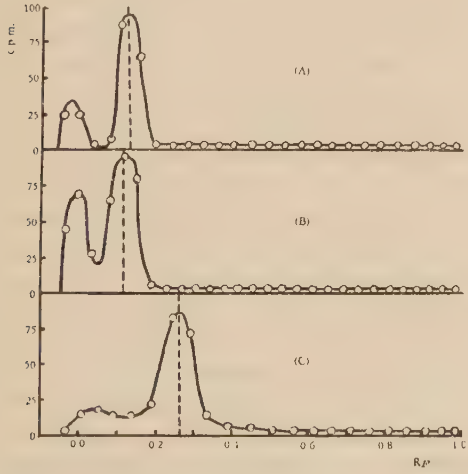

FIG. 2. Identification of Neosynthesized Organic Compounds by Paper Chromatography.

A; butanol, acetic acid and water (4:2:1)
B; butanol, ethanol and water (4:2:1)
C; isobutyric acid
Broken lines show RF values of exogenously added cystine.

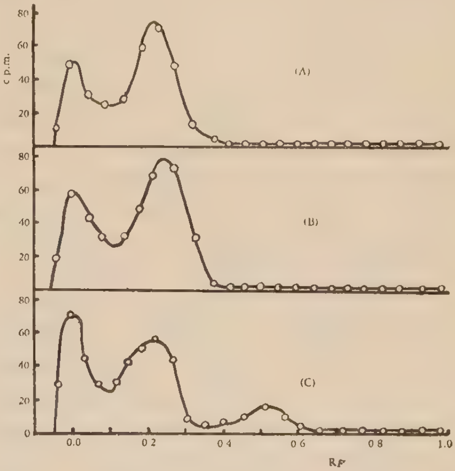

FIG. 3. Organic Compounds Neosynthesized during the Various Periods of Incubation.

A; after incubating for 1 hr. B; after incubating for 3 hr.
C; after incubating for 24 hr.

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6) G. Toennies and J.J. Kolb, *Anal. Chem.*, **23**, 823 (1951).

stance which appears to be methionine could be detected only after incubating for a long period.

DISCUSSION

As described in the introduction, in the studies on the mechanism of the conversion from sulfate-sulfur into organic sulfur in higher plants, it is indispensable that a simpler system having this biological activity is established. The results obtained from the studies on the metabolism of sulfur-containing compounds in intact plants may be very available to accomplish the above purpose. Thus, the biosynthesis of sulfur-containing organic compounds from sulfate in the seedlings was studied. As shown in Fig. 1, in the various seedlings the contents of sulfur in the organic form did not increase. In the stage of germination, the seedlings may utilize sulfur-containing organic compounds existing in the seeds as sulfur-containing materials required for growth. The results shown in Tables I, II and III illustrate these aspects in detail. The synthesis of sulfur-containing organic compounds from sulfate took place only in the leaves. Since the content of sulfur in the organic form in roots, stems and cotyledons did not exceed that of seeds and radioactive organic sulfur in these organs was detected even in the age of cultivation for 6 days after infiltrating isotope, it may be considered that sulfur-containing organic compounds in the roots and stems are transported from the cotyledons at this age. Liverman and Ragland reported recently that radioactive organic compounds were detected by the technique with paper chromatography in the roots, stems and leaves of Alaska pea seedlings which were supplied with radioactive sulfate and in excised roots radioactive organic sulfur was synthesized from radioactive sulfate[7]. Thus, in the roots and stems the synthesis of sulfur-containing organic compounds may take place in a very small scale. The most of absorbed isotope was present in the roots and stems even after cultivation for 6 days from the time when radioactive sulfate had been infiltrated. This results shows that the transport from roots to leaves takes place slowly in the seedlings. Of course, these aspects no longer hold good in plants after using the cotyledons. Thomas et al. showed that, in alfalfa, large amounts of radioactive sulfur were detected in leaves after cultivating for several days from the time when radioactive sulfate was supplied through the roots. Thus, in the adult plants, rapid transport of absorbed sulfur from roots to leaves may occur. Also, at this age, organic sulfur compounds are probably mainly formed in leaves[3].

Establishment of a simpler system capable of synthesizing sulfur-containing organic compounds from sulfate was attempted with leaves having this ability. The simplest system in the biochemical studies in the homogenizing system but the establishment of this system having the above activity was not successful. While the excised leaves, which correspond to the tissue-slices in the experiments with animals, were able to convert sulfate rapidly into the organic form, as shown in table IV, V and VI. Furthermore, great importance must be attached to rapid conversion in reduced pressure, because in this condition materials being labile in air can be employed in various experiments. Sulfite, thiosulfate and sulfide which may be intermediates in the pathway of the conversion of sulfate into the organic form are susceptible to be oxidized in air; particularly, sulfite may be rapidly oxidized in air by sulfite oxidase which is very active in this seedling[8]. In addition to the above relations, qantitative analysis of synthesized radioactive organic sulfur can be applied. The quantitative studies on the problems, whether sulfite and other possible intermediates are the true

[7] J.L. Liverman and L.B. Ragland, *Plant Phyciol.*, **31**, vii (1956).

[8] N. Harada and T. Asahi, unpublished experiment

intermediates or not, may be described elsewhere.

The radioactive substance which was detected after incubating for a short period, was cystine and it was only after incubating for a long period that the small amounts of radioactive methionine were detected. These results show that amino acid, which is first formed from sulfate, may be cystine or cysteine, while methionine may be synthesized by transmethylation from cysteine.

Acknowledgment The authors wish to express their gratitude to Professor Hidejiro Nishikawa, Professor Fujio Egami and Dr. Ikuzo Uritani for their cordial advice and helpful suggestions.

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Studies on Lignin

Part IV. Syntheses of 1-(4-hydroxy-3-methoxyphenyl)-propanediol-(1,2) and 1-(4-hydroxy-3-methoxyphenyl)-propanediol-(1,3)

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Two new phenylpropane derivatives, 1-(4-hydroxy-3-methoxyphenyl)-propanediol-(1, 2) and 1-(4-hydroxy-3-methoxyphenyl)-propanediol-(1, 3) were synthesized as lignin model compounds. The former was prepared from 2-acetoxy-1-(4-acetoxy-3-methoxyphenyl)-propanone-(1), and the latter from 3-acetoxy-1-(4-acetoxy-3-methoxyphenyl)-propanone-(1) by lithium aluminum hydride reduction, respectively. The two diols were obtained as colorless syrup, their ultraviolet spectra were determined and crystalline derivatives also prepared.

As one of the structural units in lignin, guaiacylglycerol has become prominent in recent years, Adler has synthesized guaiacylglycerol¹⁾ and its β-guaiacyl ether²⁾, and shown that the two substances recall a series of reactions typical of lignin such as sulfonation, ethanolysis and formation of formaldehyde. Lately, Freudenberg has reported that guaiacylglycerol-β-coniferyl ether was obtained as one of the intermediate products of biosynthesis of artificial lignin³⁾. On the other hand, the high reactivity of the *p*-hydroxybenzyl alcohol group, such as sulfidation with hydrogen sulfide⁴⁾ and etherification with alcoholic hydrogen chloride⁵⁾ in addition to sulfonation with neutral sulfite⁶⁾, has also been reported.

Guaiacylglycerol has three alcoholic hydroxyl groups, namely a benzyl alcohol (α-carbon atom), a secondary (β-carbon atom) and a primary (γ-carbon atom) alcohol. Of the three expected guaiacylpropanediols, 2, 3-diol is the only product which has been reported⁷⁾. In order to know the behavior of the secondary alcoholic hydroxyl group (β-carbon atom) and of the primary one (γ-carbon atom) in the presence of benzyl alcoholic hydroxyl group (α-carbon atom) independently, 1-(4-hydroxy-3-methoxyphenyl)-propanediol-(1, 2) (I) and 1-(4-hydroxy-3-methoxyphenyl)-propanediol-(1, 3) (II) have been synthesized.

Table I

(I)	$R\text{-}CHOH\text{-}CHOH\text{-}CH_3$
(II)	$R\text{-}CHOH\text{-}CH_2\text{-}CH_2OH$
(III)	$R'\text{-}CO\text{-}CHOAc\text{-}CH_3$
(IV)	$R'\text{-}CO\text{-}CH_2\text{-}CH_2OAc$
(V)	$R\text{-}CO\text{-}CH_2\text{-}CH_2OAc$

R = 4-hydroxy-3-methoxyphenyl
R′ = 4-acetoxy-3-methoxyphenyl

1, 2-diol (I) and 1, 3-diol (II) were prepared from diacetate (III) and diacetate (IV), respectively by lithium aluminum hydride reduction. Both diols obtained as syrup were sparingly soluble in ether, readily soluble in water, ethanol and acetone. Positive color reaction with diazotized sulfanilic acid shows the presence of the phenolic group, and that with quinone monochlorimide and alkali is

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1) E. Adler and S. Yllner, *Acta Chem. Scand.*, **7**, 570 (1953).
2) E. Adler and E. Eriksoo, *ibid.*, **9**, 341 (1955).
3) K. Freudenberg and H. Schlüter, *Ber.*, **88**, 617 (1955).
4) T. Enkvist and M. Moilanen, *Svensk Papperstidn.*, **55**, 668 (1952).
5) B. Leopold, *ibid.*, **55**, 816 (1952).
6) B.O. Lindgren, *Acta Chem. Scand.*, **3**, 1011 (1949).
7) K. Freudenberg and W. Heimberger, *Ber.*, **83**, 519 (1950).

a proof of the *p*-hydroxybenzyl alcoholic group[8]. The disappearance of the maximum at ca. 300 mμ in ultraviolet absorption spectrum shows a complete reduction of the carbonyl group at α-carbon atom. Ultraviolet spectra shown in Figs. 1 and 2 are the same as that of guaiacylglycerol reported by Adler and Yllner[1] and Stumpf and Rumpf[9].

Triacetate of 1, 2-diol (I) was obtained as a crystalline product having a broad melting point range. Considering the fact that the two tetraacetates of guaiacylglycerol have been successfully separated by recrystallization in a Swedish laboratory[1] and judging from our result of paperchromatography described in the experimental part of this paper, the 1, 2-diol (I) is thought to be a mixture of the two possible D, L-forms. Triacetate of 1, 3-diol (II) was not obtained as a crystalline product; however, tri-*p*-nitrobenzoate with a fairly sharp melting point was obtained. The result of paperchromatography indicates that the 1, 3-diol (II) is also a mixture of the two possible D, L-forms, one of them being in excess.

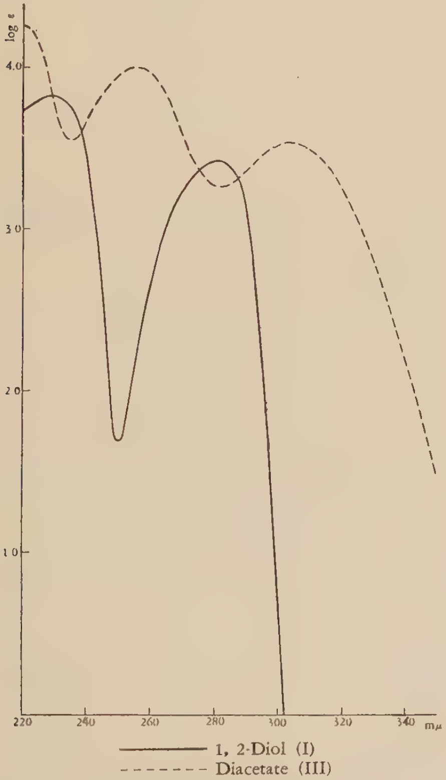

FIG. 1. Ultraviolet Spectra of 1,2-Diol (I) and Diacetate (III).

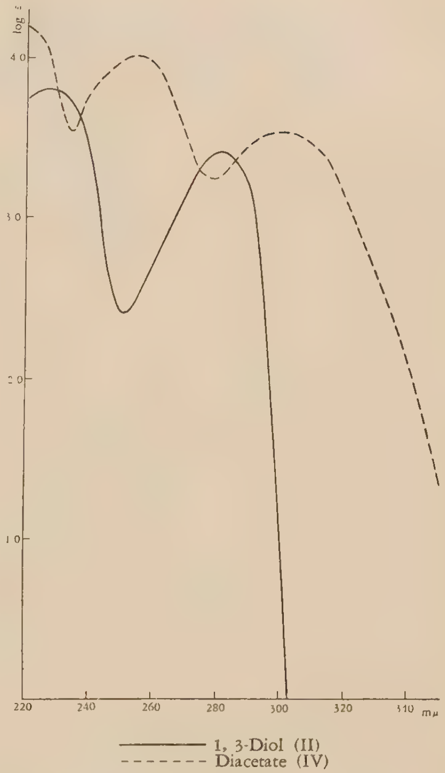

FIG. 2. Ultraviolet Spectra of 1,3-Diol (II) and Diacetate (IV)

EXPERIMENTAL

Preparation of 2-Acetoxy-1-(4-acetoxy-3-methoxyphenyl)-propanone-(1) (III). (III) was synthesized according to the method of Cramer and Hibbert[10]. m.p. 120–122°. *Anal.* Calcd. for $C_{14}H_{16}O_6$: C, 59.99; H, 5.75. Found: C, 60.11; H, 5.51.

Preparation of 3-Acetoxy-1-(4-acetoxy-3-methoxyphenyl)-propanone-(1) (IV). 3-Acetoxy-1-(4-hydroxy-3-methoxyphenyl)-propanone-(1) (V), which was synthesized according to the method of West and Hibbert[11] was acetylated with acetic anhydride and pyridine. The resulting diacetate (IV) was recrystallized from a mixture of chloroform and petroleum ether. m.p. 59–60°. *Anal.* Calcd. for $C_{14}H_{16}O_6$: C, 59.99; H, 5.75. Found: C, 59.77; H, 5.72.

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[8] J. Gierer, *Acta Chem. Scand.*, **8**, 1319 (1954).

[9] W. Stumpf and G. Rumpf, *Ann.*, **599**, 51 (1956).

[10] A.B. Cramer and H. Hibbert, *J. Am. Chem. Soc.*, **61**, 2204 (1939).

Synthesis of 1-(4-hydroxy-3-methoxyphenyl)-propanediol-(1, 2) (I). Reduction of diacetate (III) was carried out according to Nystrom and Brown[12]. A three-necked flask was equipped with a stirrer and a condenser, and between the flask and the condenser, a glass filter containing diacetate (III) (1 g) was inserted. The flask contained lithium aluminum hydride (0.5 g) and dry ether (100 ml) and the mixture was heated until the diacetate had been completely dissolved by refluxing ether (forty minutes). After a further thirty minutes of heating, the reaction mixture was cooled and water was added dropwise to decompose the excess hydride. Then, the mixture was acidified by the addition of 1 N sulfuric acid (50 ml), and extracted in a continuous extractor with ether for forty-eight hours. In the bottom of the ether flask the extracted 1,2-diol (I) was separated as a syrup. After removal of ether, the flask was washed with a small amount of acetone. The combined extract and washings were dried over sodium sulfate and a small amount of barium carbonate. Evaporation of the solvent yielded 0.7 g (98%) of colorless sticky syrup, which could not be crystallized. *Anal.* Calcd. for $C_{10}H_{14}O_4$: C, 60.59; H, 7.12, Found: C, 60.35; H, 7.07.

Synthesis of 1-(4-hydroxy-3-methoxyphenyl)-propanediol-(1, 3) (II). A solution of lithium aluminum hydride (0.5 g) in dry ether (100 ml) was placed in a three-necked flask equipped with a reflux condenser, dropping funnel and stirrer, and it was heated to produce a gentle reflux. The diacetate (IV) (1 g) dissolved in dry ether (50 ml) was added dropwise (thirty minutes). After the last addition, the mixture was refluxed for a further thirty minutes. Then, the mixture was treated in a manner similar to that described above. An amount of 0.7 g (98%) of 1,3-diol (II) was obtained as a colorless glassy mass. *Anal.* Calcd. for $C_{10}H_{14}O_4$: C, 60.59; H, 7.12, Found: C, 60.40; H, 6.89.

Paperchromatography of diols. Strips of filter paper Toyo Roshi No. 50, were treated by 0.05 M solution of sodium tetraborate, and dried. Both the borate papers and the untreated ones were saturated with water vapor and developed by the ascending method using methanol-isopropylether-water (1 : 1 : 1, upper layer)[13]. As spraying reagents, diazotized sulfanilic acid and ammonia vapor were used for the detection of phenols and quinone monochlorimide and dilute sodium hydroxide solution for the detection of *p*-hydroxybenzyl alcohols. The R_F values are listed in Table II.

TABLE II

R_F OF 1,2-DIOL AND 1,3-DIOL

	borate paper	untreated paper
1, 2-diol (I)	0.05–0.1, 0.3–0.4	0.5–0.6
1, 3-diol (II)	0.3–0.4, 0.6 (faint)	0.45, 0.55 (faint)

Preparation of Triacetates. 1,2-diol (I) was dissolved in pyridine and acetic anhydride was added. After standing overnight the mixture was poured into dilute hydrochloric acid. The resulting oil was extracted with ether. After washing with aqueous sodium carbonate and dried over sodium sulfate, the ether solution was evaporated and distilled under reduced pressure (0.3 mm, bath temp. 190°). After standing in a refrigerater for a month, the oil crystallized slowly. m.p. 59–79°. On recrystallization from an ether-petroleum ether mixture, the melting point was raised to 66–83°. *Anal.* Calcd. for $C_{16}H_{20}O_7$: C, 59.25; H, 6.22, Found: C, 59.17; H, 5.90. 1,3-diol (II) was converted to the acetate in a manner similar to that described above, but the resulting acetate could not be crystallized.

Preparation of Tri-*p*-nitrobenzoates. The diol was dissolved in pyridine and *p*-nitrobenzoyl chloride was added. After standing overnight at room temperature and at 50° for eight hours, the mixture was poured into dilute hydrochloric acid. The resulting precipitate was extracted with ether, and the ether solution was shaken with aqueous sodium carbonate. After drying and evaporation of the ether solution, the product was recrystallized from ethanol. Tri-*p*-nitrobenzoate of 1,2-diol (I) was obtained as a yellow powder. m. p. 95–101°. *Anal.* Calcd. for $C_{31}H_{23}O_{13}N_3$: C, 57.67; H, 3.59. Found: C, 57.31; H, 3.34. Tri-*p*-nitrobenzoate of 1,3-diol (II) was also a yellow powder. m.p. 84–86°. *Anal.* Calcd. for $C_{31}H_{23}O_{13}N_3$: C, 57.67; H, 3.59; N, 6.51. Found: C, 57.74; H, 3.46; N, 6.43.

Acknowledgment The authors wish to thank Miss Furusawa of this laboratory for her assistance in carrying out the microanalysis.

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[12] R.F. Nystrom and W.G. Brown, *J. Am. Chem. Soc.*, **69**, 1197 (1947).

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[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 4, p. 253~257, 1957]

Studies on Mannan in Wood Pulp

Part I. Isolatin of "Mannan" from β-and γ-Cellulose and their Sugar Composition

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Received February 27, 1957

β-Cellulose extracted from dissolving pulp with 10% aqueous sodium hydroxide, and γ-cellulose from hard sulphite pulp were fractionated into mannan, xylan and cellulose fractions according to the procedure of Hess and Lüdtke.

From the results obtained it was found that the repeating fractionations caused no increase in the mannan content being more over 40% in β-mannan fraction and 50% in γ-mannan fraction, while xylan and cellulose fractions were isolated with satisfactory results.

The purified β-mannan contained mannose, glucose and xylose at the ratio of 10 : 15 : 0.8, and the γ-mannan consisted of these sugars at the ratio of 10 : 9 : 1.

These two mannan fractions were differed from each other in the properties of their copper complex, resistances to acid hydrolysis, and degree of polymerizations.

The β-mannan was considered to consist of two parts, one of which was the degraded cellulose and the other the easily hydrolyzable γ-mannan.

Mannan is contained in the amount of about ten percent in softwoods, while, in most of hardwoods, it is one percent or less. In the case of manufacturing pulp from softwoods, an appreciable amount of mannan remains in the pulp which cannot be sufficiently removed, even in viscose or cellulose acetate manufacturing processes[1]. The turbidity[2] or abnormal viscosity[3] of the cellulose acetate solution prepared from softwood pulp has been found to be attributed to the mannan remaining in the pulp.

The chemical constructions of the mannans contained in ivory nut[4], tubers of various orchids[5], red alga[6], yeast[5] and iris seed[7] were estimated, but, as regards of the mannan of wood, the actual existence of pure mannan has remained unconfirmed since Hess and Lüdtke[8] reported a mannan isolated from sulphite pulp to be identical with ivory nut mannan A. Sherrard and Blanco[9] found that white spruce wood yielded mannose with an amount equal to or more of glucose on the hydrolysis of the extracted wood meal with 5% hydrochloric acid and no mannose was detected in the hydrolyzate in the case when 3% acid was employed. In the work of Wise and Ratliff[10], a large portion of the mannan of coniferous wood was considered more as a part of the α-cellulose rather than a part of the alkali-soluble, easily hydrolyzable hemicellulose. In addition, acetolysis of the α-cellulose indicated the fact that mannose units resided in the longer chain cellodextrins.

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[1] H. Eguchi, H. Wada, *J. Soc. Textile and Cellulose Ind., Japan*, **7**, 612 (1951).

[2] L.E. Wise, J.W. Green and R. Ritterhouse, *TAPPI*, **32**, 355 (1947).

[3] H.W. Steinmann and B.B. White, *TAPPI*, **37**, 225 (1954).

[4] M. Lüdtke, *Ann.*, **456**, 201 (1927).

[5] R.L. Whistler and L.L. Smart, "*Polysaccharide Chemistry*", p. 154 Academic Press, New York (1953).

[6] J.K.N. Jones, *J. Chem. Soc.*, 3292 (1950).

[7] P. Andrews, L. Hough and J.K.N. Jones, *J. Chem. Soc.*, 1186 (1952).

[8] K. Hess und M. Lüdtke, *Ann.*, **466**, 18 (1928).

[9] E.C. Sherrard and G.W. Blanco, *J. Am. Chem. Soc.*, **45**, 1008 (1923).

[10] L.E. Wise and I.K. Ratliff, *Arch. Biochem.*, **19**, 292 (1948).

According to Yundt[11], a mannan rich-fraction from slash pine formed crystals similar to those obtained from ivory nut mannan A. It was observed, however, that only 50% mannan was contained in this crystal, the remaining sugars being D-xylose and/or D-glucose.

It is the purpose of the present work to obtain one of the affirmations whether "mannan" present in softwood pulp is true mannan or not. An attempt to isolate the mannan from the cuprammonium solution of dissolving pulp was unsuccessful because of the too low content of mannan (6.19%) in the pulp. In this paper, β-cellulose of dissolving pulp as well as γ-cellulose of hard sulphite pulp were fractionated by Hess and Lüdtke's procedure (loc. cit.) into cellulose, xylan and mannan fractions, and their compositions, especially those of mannan fractions were studied. According to Rånby[12], α- and β-cellulose seemed to have the same crystalline lattice such as Cellulose II and, therefore β-cellulose consisted of disordered and broken micelle strings containing depolymerized cellulose. γ-Cellulose, on the other hand, was believed to be identical with hemicellulose and also with the amorphous interfibrillar substance of the pulp[13].

The fractionation of the cuprammonium solution of β-cellulose gave a small amount of xylan fraction as well as the cellulose fraction, while pure mannan could not be isolated under the applied conditions, the mannan rich fraction containing only 40% mannose. The other sugars contained in this fraction were glucose and xylose. The repeated fractionations caused no appreciable change in its composition, in spite of the remarkable depression of the fractionation yields. In the refined mannan fraction of β-cellulose (termed β-mannan to simplify the description), mannose, glucose and xylose were found in the ratio of 10 : 15 : 0.8. It was as difficult as β-cellulose to hydrolyze the β-mannan with dilute mineral acids (e.g. N H_2SO_4) or formic acid (78%), and the corresponding copper complex was insoluble in water as in the case of cellulose fraction. From the viscosity measurement of the β-mannan in cuprammonium solution, the degree of polymerization was calculated to be 150, approximately equal to that of β-cellulose.

Mannan fraction of γ-cellulose (termed γ-mannan in brief) was obtained as described above. This γ-mannan contained about 50% mannan which corresponded with the value reported by Yundt (loc. cit.). The other fraction was the xylan fraction and no cellulose fraction was contained in γ-cellulose. Just as β-mannan, γ-mannan contained glucose and xylose except mannose and the corresponding ratio of the three sugars was 9 : 1 : 10. It was easily hydrolyzed with formic acid followed by dilute mineral acid and its copper complex precipitating from the cuprammonium solution was soluble in water and formed a viscous solution. Viscosity measurements gave a D.P. of 80 for γ-mannan.

The hydrolysis under the conditions applied for β-mannan resulted in partial hydrolysis for the γ-mannan. The ratio of glucose to mannose in the hydrolyzable parts was 1 : 1 at any time in the hydrolysis stages, in spite of the fact that mannose was the main hexose of the sulphite waste liquor of soft-wood pulp[14]. The unhydrolyzable part was considered to mainly consist of the degraded cellulose.

From the fact described above, it seemed that γ-mannan is a glucomannan compounded of glucose and mannose in the approximate ratio of 1 : 1, and, in β-mannan, the degraded cellulose part is combined with or adsorbed to the γ-mannan which was considered to construct the easily hydrolyzable part of β-mannan.

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[14] L.E. Wise and E.C. Jahn, "*Wood Chemistry*" Vol. 2, p. 1019, Reinhold (1952).

EXPERIMENTAL

Preparation of β- and γ-Cellulose

A quantity of 300 g of technical dissolving pulp (manufactured at the Sanyo Pulp Mill, Japan) prepared from pine (*Pinus densiflora*) was extracted with 10% aqueous sodium hydroxide (1.8 l) for 20 hours under hydrogen atmosphere. The sludgy pulp was filtered under vacuo and washed with 10% aqueous sodium hydroxide (750 ml) into a vessel containing calculated glacial acetic acid (260 ml) with external cooling (pH., 5.9–6.0). β-Cellulose precipitated in a white amorphous state. The supernatant liquor containing γ-cellulose was removed by syphone, and then 1500 ml of water and a little toluene were added to the thick suspension of β-cellulose which was mixed throughly and allowed to stand over night. After this treatment was repeated once more the precipitate was centrifuged, filtered off the excess of water, washed with absolute methanol to free acid, then with ether, and dried in vacuo over calcium chloride. A quantity of 133 g of β-cellulose was extracted from 3 kg of the pulp.

γ-Cellulose was obtained from hard sulphite pulp which was prepared from pine (*Pinus densiflora*) with acid sulphite liquor (5.0% SO_2, 1.4% CaO) at 135° and bleached with sodium chlorite (pH 4–5) in our laboratory. A portion of 188 g of the pulp treated previously with 2% aqueous sodium hydroxide was successively extracted under hydrogen atmosphere with 800, 600 and 400 ml of 8% aqeous sodium hydroxide. The extractives were adjusted to a pH of 6.0 with glacial acetic acid as described above, and, after removal of a small amount of β-cellulose, poured into a twice amount of 95% ethanol. The amorphous precipitate of γ-cellulose was centrifuged and washed with 70% ethanol, 95% ethanol and ether. It was first dried in vacuo over calcium chloride, then over phosphorus pentoxide. The yields amounted to 4.75 g, 3.45 g and 0.50 g, respectively. The extractive with 2% aqeous sodium hydroxide (2.10 g), was found to contain only 6.71% mannan.

Distribution of Mannan in Pulp

The insoluble-part of the dissolving pulp in 10% aqueous sodium hydroxide was termed α-cellulose which was main component (91.9%) of the dissolving pulp. As shown in Table I, more amounts of mannan were contained in the lower molecular-parts of the pulp; however, two-thirds of the mannan contained in the original pulp remained in α-cellulose. In addition, even in γ-cellulose, which was the mannan richt-part of pulp, the mannan content was a half of xylan. β-Cellulose was extracted with 10% aqueous sodium hydroxide in succession, and β-cellulose-2, -3 and -4 were prepared in turn, as described on the β-cellulose (Table I). The mannan contents determined on each of the β-celluloses increased with tne proceeding of extraction to about 38% which stands closely to that of the β-mannan (see below).

TABLE I
DISTRIBUTION OF MANNAN AND PENTOSAN IN α-, β- AND γ-CELLULOSE

	Yield, %	Mannan content[d], %	Pentosan content[e], %
Dissolving pulp[a] (η_{rel}, 4.6)	—	6.19	2.66
α-Cellulose	91.9	4.04	—
β-Cellulose	4.44	11.9	5.47
β-Cellulose-2	1.65	26.5	—
β-Cellulose-3	0.32	15.5	—
β-Cellulose-4	0.07	37.9	—
γ-Cellulose	0.37	24.6	50.3
γ-Cellulose-2[f]	0.07	26.5	53.0
Sulphite pulp[b]	50.2	8.24	5.22
γ-Cellulose[c]	4.63	21.9	49.4

a) Technical sulphite pulp purified in a high degree for rayon or cellulose acetate manufacture.
b) Laboratory prepared pulp.
c) Prepared from the sulphite pulp extracted previously with 2% sodium hydroxide solution.
d) Determined by Hägglund's method.
e) By the TAPPI Standard Method.
f) γ-Cellulose extracted from α-cellulose in company with β-cellulose-2.

Colorimetric Determination of Sugars on Paper-chromatogram

No. 50 Filter paper* (20×40 cm) was used throughout these studies. A portion of 0.004 ml of a sugar solution was introduced on the filter paper at seven positions and developed by the ascending technique for 20 hours, using the solvent system of *n*-butanol-ethanol-water (40 : 11 : 19) (v/v). The development was repeated three times with drying at the end of each step. After final drying, the filter paper was cut off each other so that one strip involved one spot of sugar mixture. The separated sugar components on the alternate strips were revealed by thh spraying of aniline hydrogen phthalate. The other untreated strips were cut into small rectangles, each of which contained only one sugar. The sugars adsorbed on the filter paper were separately eluted with water and evaporated to dryness under vacuo. With the addition of water, the sugar concentrations were adjusted to the range of 10 to 50 μg/ml, and 1–ml of this solution was used for colorimetric determination. Hexoses were determined by the anthrone method[16] at 620 mμ, and pentoses, by the orcinol method[15] at 660 mμ, using a Beckman spectrophotometer. The results of the known amounts of sugars were as follows: mannose, 35.2 μg: obsv., 35.0, 35.0, 42.0 μg; glucose, 29.9 μg: obsv., 31.8, 32.0, 30.5 μg; xylose, 35.2 μg: obsv., 36.7, 36.7, 36.7 μg.

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* Manufactured by the Toyo-Roshi Filter Paper Co. Ltd., Japan

Fractionation of β-Cellulose

a) Mannan fraction (β-mannan)

To the moistened β-cellulose (3.84 g), a cuprammonium solution (100 ml) (Cu, 21.5%, NH_4OH, 28%) was added, and air-oxygen in the vessel was exchanged with hydrogen accompanified with ammonia gas and shaken until solution was complete. After 2.2 N aqueous sodium hydroxide (10 ml) was introduced, the above treatment was repeated once more and mixed throughly, and then allowed to stand for fifteen hours at room temperature. The gelatine like-precipitate, which was called the copper complex of mannan, was centrifuged. Acidification with 50% acetic acid under external cooling changed the sludgy complex of mannan to the amorphous precipitate. It was washed with 5% hydrochloric acid in methanol, absolute methanol and finally ether, and dried over phosphorus pentoxide in vacuo: yield, 510 mg.

The mannan content was determined as follows. β-Mannan (300.5 mg) was treated with 72% sulphuric acid (3 ml) at 2° and allowed to stand for 2 hours at 18 to 20° with occasional stirring. Water (100 ml) was added to this solution and it was refluxed for 4 hours. The hydrolyzate was heated with barium carbonate (10 g) until neutral and then allowed to stand overnight. The mixture was filtered and the filtrate acidified with acetic acid was concentrated to about ten ml and filtered again. Pure mannose (99.0 mg), a little sodium acetate and a phenylhydrazine acetate reagent (2 ml) were successively added to the solution. After 20 hours, the phenylhydrazone was collected and then washed with 1 ml of water, 1 ml of absolute alcohol and 2 ml of ether in this order. The yield of the phenylhydrazone derived from the mannan was 195.1 mg, m.p. 180°: this corresponds to a yield of 117.3 mg mannan, i.e., 39.1%. The other sugars contained in the mannan fraction were analyzed by paperchromatography: A neutral sugar-solution of the β-mannan (100 mg) was deionized with Amberite IR–120 and IR–4 B and concentrated to a syrup, which was paperchromatographed with standard sugars. Colorimetric determination of the sugars resulted as follows: mannose, 38.0%, glucose, 59% and xylose, 3%.

Once again, the β-mannan (1.67 g) was dissolved in the cuprammonium solution (35 ml) and reprecipitated from the solution 0.25 N in the sodium hydroxide: yield, 998 mg, mannan, 40.8%. A portion of the obtained β-mannan (750 mg) was again selectively precipitated in the same manner: yield, 358 mg, mannan, 40.8%. The presences of glucose and xylose besides mannose were shown paperchromatographically, and the results of the determination of each sugar on the paperchromatogram were: mannose, 39.5%, glucose, 58.5% and xylose, 2.0% in the second precipitate and mannose, 38.5%, glucose, 59.5% and xylose, 3.0% in the third precipitate. Uronic acids were not detected by the naphthoresorcine reaction. The mannan was insoluble in boiling formic acid (78%), dilute mineral acid or cold alkali. Repeating acetylations of the mannan dispersed previously with formamide[17] gave an unsatisfactory results.

b) Cellulose fraction

The clean supernatant, from which the copper complex of the mannan was centrifuged, was brought to precipitate by neutralization with 50% acetic acid. The sludgy precipitate was centrifuged and washed once with the cuprammonium solution, and then treated as the mannan fraction: yield, 2.33 g, mannan, 6.90%. Paperchromatography showed the presences of a large amount of glucose (88.6%) and a little xylose (5.4%) and mannose (6.0%).

c) Xylan fraction

The addition of absolute methanol twice to the centrifugate of the cellulose fraction resulted the white amorphous precipitate which was centrifuged and treated as described above: yield, 280 mg, pentosan, 74.7%. It was hydrolyzed with N sulfuric acid, and, in addition to a large amount of xylose, glucose and two oligosaccharides were detected on the paperchromatogram of the hydrolyzate. Naphthoresorcine reaction indicated the presence of uronic anhydride residues, and mannan was not contained in this fraction.

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[15] A.H. Brown, *Arch. Biochem.*, **11**, 269 (1946).
[16] R. Dreywood, *Ind. Eng. Chem. Anal. ed.*, **18**, 499 (1946).
[17] J.F. Carson and W.D. Maclay, *J. Am. Chem. Soc.*, **68**, 1015 (1946).

Fractionation of γ-Cellulose

γ-Cellulose extracted from the hard sulphite pulp was fractionated in the same way as β-cellulose already described. γ-Cellulose consisted of approximately equal amounts of xylan and mannan fractions having no cellulose fraction.

a) Mannan fraction (γ-mannan)

The copper complex of γ-mannan precipitated from the cuprammonium solution of γ-cellulose was easily soluble in water and developed into a viscous solution by acidification with 50% acetic acid. The addition of an equal volume of methanol caused the mannan to precipitate for the first time; from 6.15 g of γ-cellulose, 3.05 g of γ-mannan was obtained. The γ-mannan was selectively precipitated two times further as described above and the resultant γ-mannan (503 mg) analyzed: mannan, 50.6%. A portion of the γ-mannan (96 mg) was refluxed with formic acid (78%) for 6 hours, and then the solution was poured into a equal volume of water and evaporated to dryness. The residue was suspended in N sulphuric acid and the mixture refluxed for 1 hour. The hydrolyzate was neutralized, deionized with Amberite IR–120 and IR-4 B, concentrated to a syrup and paperchromatographed. Determinations of the component sugars were: mannose, 50.0%, glucose, 44.9%, and xylose, 5.1%. The mannan was insoluble in water, alkali and dilute mineral acids.

b) Xylan fraction

The centrifugate of the γ-mannan produced no precipitates by neutralization with 50% acetic acid, and therefore, two volumes of absolute methanol was added to the solution and treated as xylan fraction of β-cellulose. The twice repeated precipitations resulted xylan (1.80 g) containing 75.1% pentosan and 20.1% uronic acids. Xylose and two oligosaccharides were detected on the paperchromatogram.

Partial Hydrolysis of β-Mannan

Heating the β-mannan (100 mg) with formic acid (78%) or N sulphuric acid (5 ml) in a sealed tube at 110° resulted partial hydrolysis: In the case of N H_2SO_4, 30.6% (1 hr.), and 52.7% (8 hrs.); in the case of formic acid, 40.6% (5 hrs.), 66.0% (10 hrs.), 67.2% (10 hrs., for the 2nd. precipitate) and 67.0% (10 hrs., for the 3rd. precipitate). Hydrolysis with formic acid was followed by the treatment of N sulphuric acid, the last condition giving maximum reducing power to the mannan. The determinations of glucose and mannose on the paperchromatogram are given in Table II, which shows that an equal amount of each sugar is yielded from the β-mannan at any stage of the hydrolysis process. Amounts of xylose contained in the hydrolyzates were 10.5%, 5.7% and 3.0% at the rate of hydrolysis of 30.6%, 52.7% and 91.2%, respectively. An insoluble part of the β-mannan (ca. 20%) on heating with the formic acid for 10 hours was centrifuged and completely hydrolyzed with 72% sulphuric acid. A large amount of glucose as well as traces of xylose and mannose was detected on the paperchromatogram.

TABLE II

THE AMOUNTS OF GLUCOSE AND MANNOSE FOUND IN THE HYDROLYZATES OF β-MANNAN

Rate of hydrolysis[a], %	Glucose, μg.	Mannose, μg.	Ratio of mannose to glucose
1st Precipitate			
30.6	15.8	18.0	1.14
40.6	49.0	47.5	0.97
52.7	27.3	26.7	0.98
66.0	46.0	45.0	0.98
2nd Precipitate			
67.2	40.7	42.5	1.04
3rd Precipitate			
67.0	30.5	31.2	1.02

a) Determined by iodometry.[20]

Viscosity Measurement

The viscosity of each fraction was measured on the cuprammonium solution (0.1% concentration). The intrinsic viscosity was calculated from the relative viscosity using the Schultz and Blaschke formula[18] and K values observed by Huseman[19], were applied to the present experiment, namely 4.4×10^{-4} to the mannan fractions and 5.0×10^{-4} to the others. Table III, shows the degree of polymerizations (D.P.) calculated from relative viscosity.

TABLE III

D.P. VALUES OF VARIOUS FRACTIONS

Kind of fraction	D.P. values
β-Mannan	150
γ-Mannan	80
Xylan[a]	50
Cellulose	100
β-Cellulose	140
γ-Cellulose	50

a) Isolated from γ-cellulose.

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[18] G.V. Schulz und F. Blaschke, *J. prakt. Chem.*, **158**, 136 (1941).

[19] E. Huseman, *J. prak. Chem.*, **155**, 13 (1940).

[20] J.L. Baker, *Biochem. J.* **14**, 754. (1920).

Short Communications

On the Reduction Products of Rotundifolone with Lithium Aluminum Hydride

(Studies on the Essential Oil of *Mentha rotundifolia*, Part III*)**

Sir:

Rotundifolone, the principal component of the oil of Japanese *Mentha rotundifolia*, was established to be 1-methyl-4-isopropylidene-1, 2-epoxy-cyclohexanone-3 in our previous report.* It has also been pointed out that "mintglyoxal" isolated from Indian *Mentha viridis*, was presumably identical with rotundifolone, but the glyoxal structure proposed by Chakravarti and Bhattacharyya1) can not be accepted as that for rotundifolone***. Independently, Reitsema2) isolated the same substance as ours, and he has also indicated that rotundifolone was identical with "lippione", which had been isolated from the oil of Argentine *Lippia turbinata* by Fester et al3).

We obtained dihydro-rotundifolone by the reduction of rotundifolone over palladium, while Reitsema did not succeed in hydrogenation of rotundifolone by the use of a platinum catalyst. Our dihydro-rotundifolone semi-carbazone showed no depression, upon admixture with *l*-piperitone oxide semicarbazone which Reitsema and Varnis4), isolated from the American *Mentha sylvestris*.

As regards to the reduction product of rotundifolone by lithium aluminum hydride, Reitsema has assumed it to be 2,3-diol (II)2) on the basis of its positive periodic test. In our previous report,* we gave 1, 3-diol structure (II a, b) for two stereoisomers of the diol of m.p. 145° and of m.p. 85°. Neither of the dihydro-diols (III a and b), obtained by the catalytic hydrogenation of diols (II a, b) over palladium gave a positive periodic acid test. Dihydro-diol (III a) from diol of m.p. 145° (II a) is a levorotatory needle, m.p. 165°, $[\alpha]_D^{15}-33.0°$, and the one (III b) from diol of m.p. 85° (II b) a dextrorotatory oil, $[\alpha]_D^{10}+20.35°$, d_D^{10} 0.975, n_D^{10} 1.4712.

Macbeth and Robertson5), have determined the configuration of various stereoisomers of *p*-menthane-diol-2, 3, but neither of our dihydrodiols coincides with any stereo-isomer of their 2, 3-diols.

Recently, Chatterjee and Majumder6) reported that periodic acid was a promising reagent for the detection of both terminal and exocyclic double bonds. Consequently, in such diols as (II a, b), which possess an exocyclic double bond, a positive periodic reaction does not necessarily indicate the presence of α-glycol grouping.

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* Part II, This Bulletin, **21**, 107 (1957).

** This report was presented at the Annual Meeting of Agric. Chem. Soc. Japan, Univ. of Tokyo, April 11, (1957).

1) K.K. Chakravarti and S.C. Bhattacharyya, *P&E.O.R.* **46**, 256 (1955).

2) R.H. Reitsema, *J. Am. Chem. Soc.*, **78**, 5022 (1956).

3) G.A. Fester, E.A. Martinuzzi and A.I. Riccardi, *Rev. fac. ing. quim.*, **21**, 43 (1952), *C.A.* **48** 6655 (1954); *Anales asoc. Quim. argentina*, **42**, No. 2, 43 (1954), *C.A.* **49** 3479 (1955).

*** We received mintglyoxal derivatives from S.C. Bhattacharyya on May 5, 1957. When mintglyoxal semicarbazone and two stereoisomeric diols (m.p. 80° and 146°), which were obtained by lithium aluminum hydride reduction, were mixed with our corresponding samples from rotundifolone, respectively, they melted with no depression in each case. Moreover, in his personal communication to us, three properties of mintglyoxal given in his previous report1) were corrected as the following, so, it has been established that mintglyoxal is completely identical with our rotundifolone:

a) The ferric chloride test of mintglyoxal was positive→negative, after three recrystallizations;

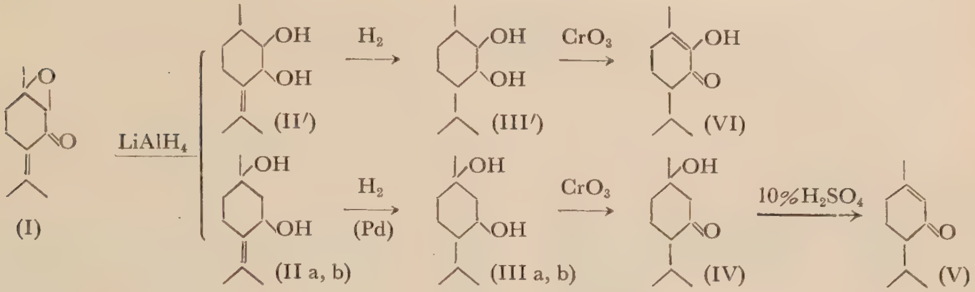
b) λ_{max} of semicarbazone, 250 mμ→273 mμ;

c) Lithium aluminum hydride reduction product, diol of m.p. 148°→a mixture of two diols, m.p. 80° and m.p. 146°.

4) R.H. Reitsema and J. Varnis, *J. Am. Chem. Soc.*, **78**, 3792 (1956).

5) A.K. Macbeth and W.G.P. Robertoson, *J. Chem. Soc.* 3512 (1953); 701 (1954).

6) A. Chatterjee and S.G. Majumder, *Anal. Chem.*, **28**, 878 (1956).



As the tertiary hydroxyl group resists to chromic acid, *p*-menthane-1, 3-diol (III) will yield 1-hydroxy-menthone (IV) by this oxidation, whereas, *p*-menthane-2, 3-diol should give diosphenol (VI), which is easily detectable by the ferric chloride reaction and the intense absorption maximum at 273 mμ.

The chromic acid oxidation products of (III a, b) were the oil which were assumed to be the mixture of 1-hydroxy-menthone (IV) and a small amount of piperitone (V). When oil (IV), was refluxed with 10% sulfuric acid solution for 2 hrs., *dl*-piperitone (V) was obtained almost quantitatively. 2, 4-Dinitro-phenylhydrazone of (V) is a red crystal, melting at 118°. *Anal.* Found: C, 57.70; H, 6.30. Calcd. for $C_{16}H_{20}O_4N_4$: C, 57.82; H, 6.70%. It showed no depression of melting point when mixed with the corresponding derivative of an authentic *dl*-piperitone, isolated from the Japanese peppermint oil. Semicarbazone of (V) was a crystal, hardly soluble in methanol, melting at 214°. It also did not depress the melting point upon admixture with an authentic *dl*-piperitone semicarbazone.

By lithium aluminum hydride reduction, a secondary oxide linkage is attacked in preference to a tertiary, and tertiary alcohol is produced, although some exceptions have been reported. In the case of rotundifolone, the formation of *p*-menthane-1, 3-diol is dominant. The synthesis of piperitone from rotundifolone does not only support the presence of an epoxide linkage, but also establishes the relation between rotundifolone and menthol series.

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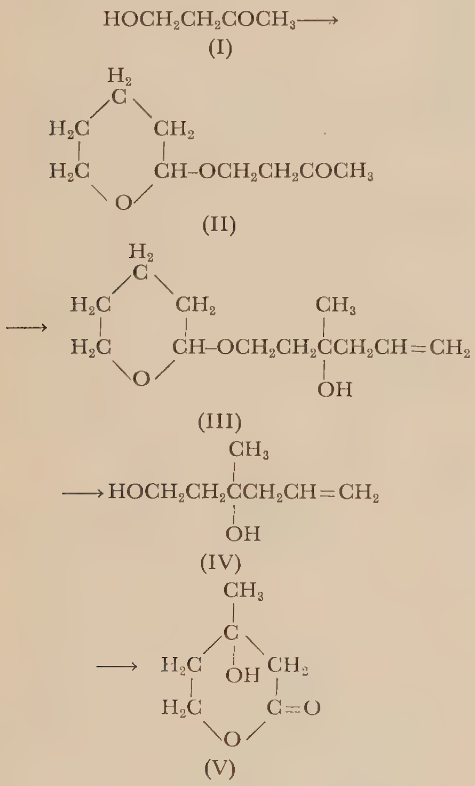
Received May 18, 1957

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 4, p. 260, 1957]

A New Synthesis of DL-β-Hydroxy-β-methyl-δ-valerolactone

Sir:

Wolf, et al.[1], have recently shown that β-hydroxy-β-methyl-δ-valerolactone (V) is a new acetate-replacing factor for lactobacilli and have reported the synthesis of the DL-compound by partial reduction of diethyl β-hydroxy-β-methylglutarate. In this paper we wish to describe a new synthesis, which differs from the above method.



1-Hydroxy-3-butanone (I), b.p. 65–68°/8 mm, prepared from acetone and paraformaldehyde[2], was treated with 2,3-dihydropyrane containing a few drops of concentrated hydrochloric acid to give 2-(2-acetoethoxy)-tetrahydropyrane (II), b.p. 97–98°/5 mm, in a 77% yield. *Anal.* Calcd. for $C_9H_{16}O_3$: C, 62.76; H, 9.36. Found: C, 62.61; H, 9.42[3]. When this tetrahydropyranyl ether and allylbromide were added to Mg in dry ether, 2-(3-allyl-3-hydroxybutoxy)-tetrahydropyrane (III), b.p. 103–104°/2 mm, was obtained in a 55% yield. *Anal.* Calcd. for $C_{12}H_{22}O_3$: C, 67.25; H, 10.35. Found: C, 67.49; H, 10.52. III was refluxed gently with dilute hydrochloric acid to afford 3-allyl-1,3-butanediol (IV), b.p. 102–104°/5 mm, in a 77% yield. *Anal.* Calcd. for $C_7H_{14}O_2$: C, 64.58; H, 10.84. Found: C, 64.10; H, 11.20. IV was ozonized in ethyl acetate and the resulting ozonide converted directly into β-hydroxy-β-methyl-δ-valerolactone (V) with hydrogen peroxide in acid solution. Thus, the valerolactone, b.p. 148–151°/5 mm, was obtained as a viscous syrup in a 51% yield. *Anal.* Calcd. for $C_6H_{10}O_3$: C, 55.37; H, 7.75. Found: C, 55.12; H, 7.43. The infra red spectrum of this substance in liquid film is consistent with the structure V, having characteristic bands at 1730 cm^{-1} (δ-lactone) and 3448 cm^{-1} (hydroxyl). It also showed biological activity toward *Lact. heterohiochi*[4].

Synthesis of its homologs are now being further investigated.

We wish to express our sincerest thanks to Prof. Y. Sumiki for his guidance and encouragement through these experiments.

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Received May 24, 1957

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[1] D. Wolf, C. Hoffman, P. Aldrich, H. Skeggs, L. Wright and K. Folkers, *J. Am. Chem. Soc.*, **78**, 4499 (1956); D. Wolf, C. Hoffman, P. Aldrich, H. Skeggs, L. Wright and K. Folkers, *ibid.*, **79**, 1486 (1957).

[2] T. White and H. Harward, *J. Chem. Soc.*, **1943**, 25.

[3] The microanalyses reported herein were carried out by members of the Analytical Laboratory of this Department, to whom we are grateful.

[4] We are indebted to Mr. G. Tamura of this Department for the biological test.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 4, p. 261～262, 1957]

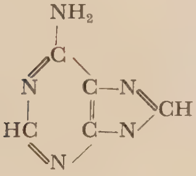
On the Studies of Angustmycins

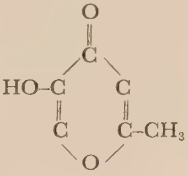
IV. Degradation Studies of Angustmycin A

Sir:

In the previous report1), the existence of adenine as a structural fragment in angustmycin A was indicated spectrographically, while the other moiety was presumed to be a sugar. The acid cleavage of the antibiotic under mild condition yielded adenine (I), and a new sugar (II), to which the name angustose was proposed. However, under a more vigorous condition, instead "angustose", allomaltol (III) was obtained, which was proved to be an acid decomposition product of angustose.

The characterization and identification of these three compounds are reported in this paper.


(I)


(III)

When angustmycin A was subjected to hydrolysis with 1/10 N hydrochloric acid in a boiling-water bath for 30 minutes, the hydrolysate gave a positive intensive Fehling, ammoniacal silver nitrate reactions. On treatment of the hydrolysate with cation exchange resin Amberlite IR 120, successively with the anion exchange resin Amberlite IR 4 B, a neutral solution was obtained, which was concentrated in vacuo to yield a pale yellow syrup. When this syrup was further purified by cellulose powder partition chromatography with water saturated butanol, a crystalline substance of melting point 116–118° (dec.), $[\alpha]_D^{20}$ 18° (c, 1% in ethanol) was obtained. The tentative formula $C_6H_{10}O_5$ was derived from elemental analysis. *Anal.* Calc. for $C_6H_{10}O_5$: C, 44.44; H, 6.22. Found: C, 44.18; H, 6.40.

The crysralline substance gave positive Fehling, Molish, ammoniacal silver nitrate, anthrone and iodoform reactions.

The Kuhn-Roth determination indicated the presence of one C-CH_3 group. The infrared absorption spectrum exhibited bands at 3450 cm^{-1}, 1710 cm^{-1}, 1210 cm^{-1}, 1160 cm^{-1}, 1120 cm^{-1}, 1066 cm^{-1}, 1040 cm^{-1}, 935 cm^{-1}, 910 cm^{-1}, and 780 cm^{-1}. On the basis of these results this substance was considered to be a natural occurring unknown sugar, named angustose. From the concentrated eluate of the cation exchange resin Amberlite IR–120 with 1 N hydrochloric acid, a crystalline needle was obtained as the hydrochloride 1/2 hydrate, which melted at 284–286° (dec.), and showing one peak of U.V. at 260 mμ. *Anal.* Calc. for $C_5H_5N_5HCl\cdot{}^1/_2H_2O$: C, 33.2; H, 3.9: N, 38.8; Cl, 19.6. Found: C, 33.3; H, 3.8; N, 38.7; Cl, 19.55.

On treatment of this crystalline hydrochloride in aqueous solution with the anion exchange resin to remove hydrochloric acid, the free base was obtained as needles, which decomposed at the range over 350° (in an air bath) showing one absorption peak of U.V. at 260 mμ. (ε 13000). *Anal.* Calc. for $C_5H_5N_5$: C, 44.44; H, 3.73; N, 51.83. Found: C, 44.38; H, 3.87; N, 51.83.

The identity of this crystalline base with adenine was confirmed by the coincidence of a IR spectrum.

Under a more acidic condition, such as with 1 N hydrochloric acid in a boiling-water bath, the hydrolysate showed an intense reddish color for 10 minutes, and almost all the

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1) Hsü Yüntsen, K. Okuma, Y. Ishii and H. Yonehara,

angustose was found to be decomposed by the disappearance of its papergraphic spot at R_F 0.40. (butanol : acetic acid : water, 4 : 1 : 5, v/v).

In addition to this fact, the appearance of a new spot at R_F 0.82 was recognized. The hydrolysate was extracted with ether at pH 2, from which a crystalline substance was obtained. It sublimed at 110°, melted at 154–156°, and gave an intense reddish-brown color with $FeCl_3$. *Anal.* Calc. for $C_6H_6O_3$: C, 57.14; H, 4.76. Found: C, 57.30; H, 4.78.

It was a weak acidic substance of pka. 8.25, and showed one U.V. absoption peak at 267 mμ in 1/10 N HCl (ε : 9800) which shifted to 307 mμ in 1/10 N NaOH. (ε : 5700). The IR spectra exhibited characteristic bands of α, β, α′ β′- unsaturated keton at $1665\,cm^{-1}$, that of $>C=C<$ at $1610\,cm^{-1}$.

The Kuhn-Roth determination gave one equivalent of $C-CH_3$. On the basis of these data, it was presumed to be one of the pyrones, and the identity with allomaltol (2-methyl-5-hydroxy-4-pyrone) was confirmed by no depression of mixed melting point as well as the coincidence of IR spectrum with that of an authentic specimen.

The formation of allmaltol from angustose was proved by the degradation of the latter with 1 N hydrochloric acid, but the yield was not quantitative, being approximately 23% by spectrophotometrical determination.

Further work is now in progress and will be reported in a recent paper.

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The Journal of Antibiotics. Ser. A, 9, 195 (1956).

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Accumulation of Isocitric Acid by a *Penicillium* Strain

Sir:

The accumulation of isocitric acid from glucose by *Penicillium purporogenum* Stoll *var. rubri-sclerotium* Thom (non-sclerotigenic type) no. 1148[1] in medium was proved by the isolation and purification of the above acid. This acid, known as one of the members of the TCA cycle, was found to be contained in some higher plants, in considerable amounts[2,3]. Although the occurrence of the acid in the TCA cycle of bacteria has been proved, the accumulation of this acid by microorganisms has, so far, never been reported.

This fungus was grown aerobically in a medium containing 10% glucose, 0.3% peptone and 1% $CaCO_3$ by shaking culture. From the culture filtrate, the acid was extracted with ether and the extract evaporated to a syrup which was then heated at 100° in vacuum so as to dehydrate the acid to its γ-lactone. The lactone was purified by recrystallization from ethylacetate and chloroform.

The γ-lactone, γ-lactone-dimethylester, and tri-acid-amide were obtained in the crystalline form.

The lactone was titrated as a dibasic acid in the cold, but after treatment with excess alkali, one more acidic group appeared; it melted at 140–141° to yellow oil.

Anal. Calculated for $C_6H_6O_6$: C, 41.33; H, 3.47. Found: C, 41.24; H, 3.54.

$[\alpha]_D^{19°}$; +42.3° (c=4.83, H_2O)

Dimethyl ester of lactone: m.p. 49–50°

Anal. Calculated for $C_8H_{10}O_6$: C, 47.53; H, 4.99. Found: C, 47.80; H, 5.34.

$[\alpha]_D^{22°}$; +56.2° (c=0.85, MeOH)

Tri-acidamide, m.p. 189° with decomposition.

Anal. Calculated for $C_6H_{11}O_4N_3$: C, 38.09; H, 5.88; N, 22.20. Found: C, 38.27; H, 5.58; N, 21.70.

The infrared spectrum of tri-sodium salt of the isolated acid was almost completely identical with that of an authentic specimen. (Fig 1, 2)

By dry distillation of the lactone, citraconic and itaconic anhydrides were obtainnd. The former was identified by paper chromatography. The latter acid was purified from this distillate by silica gel chromatography. Admixture with authentic itaconic acid showed no melting point depression.

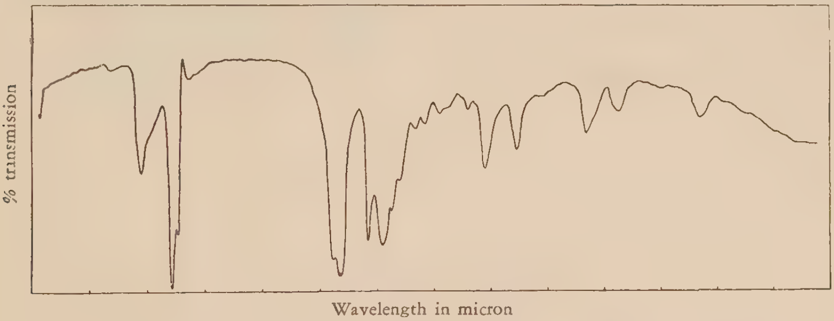

FIG. 1. Infrared Spectrum of the Tri-sodium Salt of the Isolated Acid in Nujol mull.

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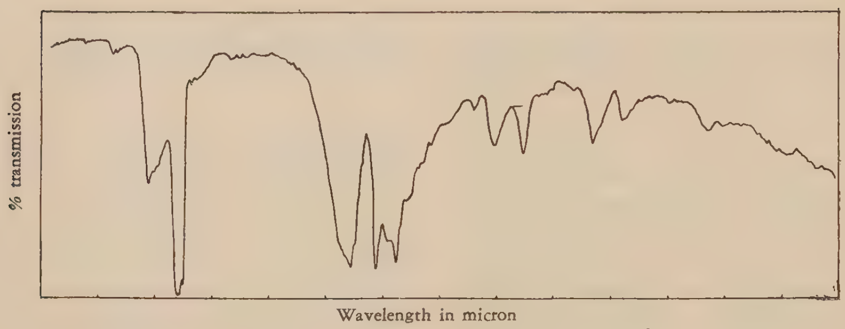

FIG. 2. Infrared Spectrum of the authentic Tri-sodium Isocitrate in Nujol mull.

However, as shown in the melting points and $[\alpha]_D$ values, the isolated acid was far different from the ordinary optically active isocitric acid which was found to occur in the TCA cycle. Therefore, it is supposed that the isolated acid might be the diastereoisomer of isocitric acid, i.e., allo-isocitric acid. This was supported by the result of paper chromatography with the synthetic product containing a considerable amount of the allo form which was synthesized by the method originally described by Fittig-Miller[4,5]. The paper chromatographic behavior of the isolated acid and the synthetic allo-acid were completely identical. (Table I)

From the above results, it was concluded that the isolated acid should be allo-isocitric acid. The prsesence of allo-isocitric acid in nature, has yet never been reported.

TABLE I
PAPERCHROMATOGRAPHIC IDENTIFICATION OF ALLO-ISOCITRIC LACTONE

solv: *n*-BuOH-formic acid-water (4 : 1.5 : 1 v/v)

R_F value of	the isolated lactone	0.69
〃	the synthetic product containing allo-acid (three spots)	0.69 0.60 0.91*
〃	*dl*-isocitric lactone	0.60

*Small amount, probably due to polymerization.

The yield of isocitric acid in weight, in some cases, exceeded 20% of consumed glucose. There were no other acids detectable in the culture medium.

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[4] R. Fittig and H.E. Miller, *Ann. Chem. Justus Liebigs*, **255**, 43 (1889).

[5] G.W. Pucher and H.B. Vickery, *J. Biol. Chem.*, **163**, 169 (1946).

*Kyowa Fermentation Ind. Co. Ltd.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 4, p. 265, 1957]

Resolution of (±)-*trans*-3-(*trans*-2′-carboxypropenyl)-2,2-dimethylcyclopropane-1-carboxylic Acid

Sir:

The partial resolution of (±)-*trans*-3-(*trans*-2′-carboxypropenyl)-2,2-dimethylcyclopropane-1-carboxylic acid1) by means of a synthetic optical active (−)-α-phenylethylamine to give the dextrorotatory acid, which was completely identical with the naturally derived acid, has already been descrived by the authors, and here, the complete resolution was achieved by obtaining the enantiomorphic laevorotatory acid with the results summarized below (rotations in ethanol).

	m.p.	$[\alpha]_D^{11}$
(+)-acid	163–4°	+70.9°
(±)-acid	206–8°	0
(−)-acid	163–4°	−70.5°

The combined filtrate from (−)-base (+)-acid salt1) was evaporated, decomposed with dilute sulphuric acid and recovered by extraction. To a boiling solution of the recovered acid (3.4 g) in 80 ml of methanol, was added 2.1 g of (+)-α-phenylethylamine, having $[\alpha]_D^{11}+39.4°$, dissolved in 15 ml of methanol. After the solution was kept overnight the first crop (1.4 g) was obtained and the mother liquor concentrated, this procedure being repeated so that four crops were obtained, the rotations of which varied in a range from −15° to +3°. After successive recrystallizations and by combining crops with approximately the same rotation and crystallizing them as many times as before, there was finally obtained a pure (+)-α-phenylethylamine salt of the (−)-acid (0.6 g) m.p. 223–4°, $[\alpha]_D^{11}-28.0°$ (*c*, 1.1, methanol) (*Anal.* Found: C, 67.93; H, 8.08; N, 4.28, Calcd. for $C_{10}H_{14}O_4 \cdot C_8H_{11}N$: C, 67.69; H, 7.89; N, 4.39) Further recrystallization did not alter these values. The salt (0.5 g) was decomposed by pouring an aqueous solution into a dilute sulphuric acid, and extraction and recrystallization gave the pure (−)-acid (0.26 g) m.p. 163–4°, $[\alpha]_D^{11}-70.5°$ (*c*, 1.0, ethanol) (*Anal.* Found: C, 60.87; H, 7.28, Calcd. for $C_{10}H_{14}O_4$: C, 60.59; H, 7.12) *Di-p-phenylphenacylester* m.p. 151–2°, $[\alpha]_D^{11}-123.0°$ (*c*, 0.91, chloroform) (*Anal.* Found: C, 77.97; H, 6.05, Calcd. for $C_{38}H_{34}O_6$: C, 77.79; H, 5.84).

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Additions and Corrections

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page	column	line	
277	TABLE II		printed :

pH		log I_0/I (450 m)× dilution	pH		log I_0/I (450 m)× dilution
min.			min.		
0	60		0	60	

corrected :

without phosphates			with phosphates		
pH		log I_0/I (450 mμ)× dilution	pH		log I_0/I (450 mμ)× dilution
min.			min.		
0	60		0	60	

page	column	line	
283	left	17—18	printed :

$[\alpha]^{27}_{\mathrm{D}}$ $-6.7 \rightarrow +2.8$

corrected :

$[\alpha]^{27}_{\mathrm{D}}$ $-67^\circ \rightarrow +28^\circ$

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page	column	line	
75	left	ref. 3)	printed : (1940) corrected : (1941)

Corrected Figure

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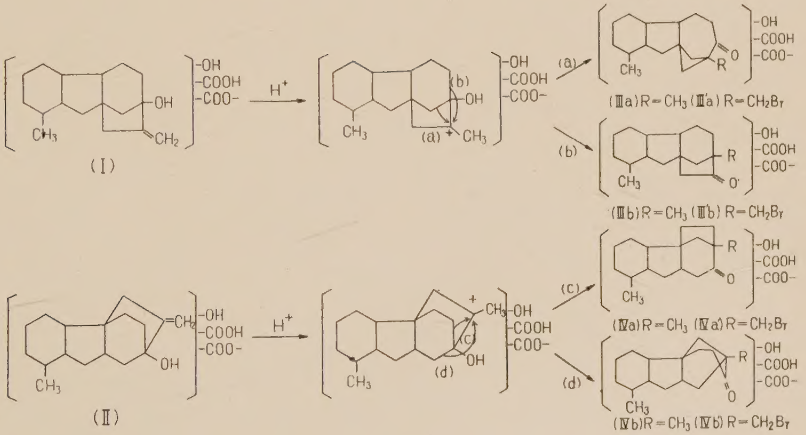
-OH
-COOH
-COO-
CH3
CH2
(I)
H+
(a)
(b)
(c)
(d)
CH3
R
O
(IIIa) R=CH3 (III'a) R=CH2Br
(IIIb) R=CH3 (III'b) R=CH2Br
(IVa) R=CH3 (IV'a) R=CH2Br
(IVb) R=CH3 (IV'b) R=CH2Br
=CH2
OH
(II)

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